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14. ABSTRACT
In the C3(1)/SV40 T-antigen (Tag) FVB/N mouse model of human estrogen and progesterone receptor-negative breast cancer, the stress response elicited by social isolation is associated with increased expression of metabolic genes in the mammary gland. To further understand accelerated tumor growth associated with social isolation, we separated mammary gland adipocytes from ductal epithelium and stroma and then analyzed individual fractions for changes in metabolic gene expression and function. The increased expression of the key metabolic genes Acaca, Hk2 and Acly was found to be significantly elevated in the adipocytes of the mammary gland, and surprisingly, was not significantly increased in visceral adipose depots of socially isolated female mice. Increased metabolic gene expression in the mammary gland of socially isolated mice coincided with increased glucose metabolism, lipid synthesis, and leptin expression. Furthermore, culture media from isolated versus group-housed mouse mammary adipose tissue resulted in relatively increased proliferation of mammary cancer cells. These results suggest that exposure to chronic social isolation results in metabolic changes in mammary gland adipocytes that contribute to increased growth of adjacent epithelial cell tumors. We propose a model in which environmental stress affects estrogen-independent mammary tumor growth, at least in part, through changes in mammary adipocyte biology.

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Introduction

We previously identified key lipid synthesis genes including acetyl-CoA carboxylase alpha (*Acaca*) and ATP citrate lyase (*Acly*) as significantly over-expressed in the mammary adipocytes from isolated vs. group-housed TAg mice. Increased expression of these metabolic genes was also associated with increased glucose consumption and increased lipid synthesis in mammary adipocytes. Furthermore, conditioned media from mammary adipose tissue of socially isolated animals potentiated the proliferation of an SV40 TAg mammary cancer cell line. These data identify an association between chronic social stress, altered mammary fat metabolism, and the secretion of tumor promoting factors from the mammary adipocytes of TAg mice. Ongoing studies have focused on the metabolic alterations within the mammary adipocytes of socially isolated mice. In particular, we have identified significant changes in mammary fat lipid species within the social isolates. Our current investigations test the hypothesis that pro-tumorigenic lipid species are synthesized in the mammary adipocytes of socially isolated mice and contribute to increased tumor burden.

Human epidemiological studies have revealed that social isolation is associated with an increased risk of both all-cause mortality and metabolic diseases such as diabetes (1). Although association studies examining social isolation and human cancer risk have had mixed results (2, 3), the conclusions of these studies are likely inconsistent because of the genetic and environmental variation inherent in human populations as well as the extensive heterogeneity of breast cancer subtypes (4). These issues make identifying underlying mechanisms connecting social stressors to breast cancer biology challenging. They also underscore the importance of developing well-defined preclinical models for identifying the variety of connections between social stressors and specific cancer subtypes.

In female rodents, imposed social isolation is a well-defined chronic stressor and has been associated with increased mammary tumor growth and malignancy within Sprague-Dawley rats and increased tumor burden in the SV40 T-antigen (TAg) FVB/N mouse breast cancer model (5, 6). Interestingly, increased tumor burden and increased tumor invasiveness occur independently of circulating estrogen and progesterone levels. Global gene expression data from our laboratory previously suggested a link between stress-induced mammary gland lipid synthesis gene expression and subsequently increased mammary tumor growth in the TAg model of ER-negative human breast cancer (6).

Our latest manuscript (appendix, section 2) reports that the increased metabolic gene expression is specific to the mammary adipocytes from socially isolated mice and is associated with a concomitant increase in mammary adipocyte glucose and lipid metabolism. Interestingly, the upregulation of *Acaca, Hk2,* and *Acly* steady-state mRNA that was involved in lipid synthesis, was not observed in the visceral fat depots of either FVB/N (both SV40-TAg and wild type) or CD1 female mice. This suggests that stress-induced changes in metabolic gene expression are mammary adipocyte-specific and not dependent on the animal strain. Furthermore, mammary adipocytes from socially isolated versus group-housed mice exhibited increased leptin production. When mammary fat was cultured, media from isolated animals' fat had significantly higher levels of secreted leptin and also stimulated SV40-TAg epithelial cell growth to a greater extent than media from group-housed adipose tissue. These results support the hypothesis that secreted factors from metabolically altered mammary adipose tissue contribute to larger tumor formation in socially isolated versus group-housed mice (appendix, section 1).

Our ongoing investigations have focused on the metabolic alterations within the mammary adipocytes of socially isolated mice. In particular, we have identified upregulation of the lipid synthesis-regulating transcription factor, ChREBP, in the mammary fat of socially isolated animals. Furthermore, lipidomics profiling of mammary lipids from isolated vs. grouped animals has revealed significant increases in specific

polar and neutral lipid species within the mammary fat of social isolates. Among these lipids, lysophosphatidylcholine (LPC), the major precursor to the important signaling lipid, lysophosphatidic acid (LPA), has been investigated as a lipid species that may contribute to the increased tumor burden in social isolates. Interestingly, whereas physiological LPC doses were toxic to TAg breast cancer cells and sub-physiological doses inhibited TAg cell proliferation, in the presence of mammary fat conditioned media, LPC protected TAg breast cancer cells from apoptosis and enhanced their proliferation. Furthermore, exogenous LPA protected TAg breast cancer cells from apoptosis and increased cell proliferations, suggesting that LPC acts as an intermediate metabolite with pro-tumorigenic effects upon conversion to LPA. These data identify LPC and LPA as potentially important lipid signaling species involved in the increased tumor burden observed in socially isolated female TAg mice.

The lipid synthesis-regulating ChREBP α/β genes are differentially regulated in the mammary fat of social isolates.

Upon a meta-review of microarray data comparing grouped and socially isolated animals' mammary fat pad gene expression, *MLXIPL* was identified as significantly increased in social isolates and also encoding for a transcription factor (ChREBP) reported to coordinate lipid synthesis pathways. ChREBP (α) mRNA upregulation in social isolates was validated with qPCR (Fig. 1A). Interestingly, we also observed significant mRNA upregulation of ChREBP-β, a novel ChREBP isoform recently identified in adipose tissue (7). To further characterize mRNA level changes to glucose metabolism and lipid synthesis genes, we performed qPCR on the glucose transporter gene (*GLUT1*) and several other genes involved in the lipid synthesis pathway (*FASN, SCD1, ELOVL6*). Of these genes profiled, *GLUT1* and *ELOVL6* were also significantly increased (Fig. 1A). Upregulation of these genes provide further validation for activated glucose metabolism and lipid synthesis pathways in the mammary fat of social isolates (Fig. 1B) and identify ChREBP as an important transcription factor potentially involved in coordinating the metabolic alterations in mammary adipocytes.

Increased polar lipid classes in the mammary fat of socially isolated animals.

The ChREBP gene product has been understudied in adipose tissue; however, recent investigations have suggested that it could play important roles in regulating systemic glucose metabolism through coordinating lipid synthesis in the adipocytes (8). The products of lipid synthesis in adipocytes include neutral lipids that can be stored as triglycerides and polar lipid species that incorporate the products of *de novo* lipid synthesis into their glycerol backbone. Because many of these polar lipids have important signaling roles, some of which are known to influence cancer biology, we performed mass spectrometry-based lipidomics to determine whether increased lipid

synthesis in the mammary adipocytes of socially isolated animals coincides with changes to specific polar lipid classes and/or species.

Lipidomics profiling was performed on polar lipids extracted from the mammary fat pads of TAg mice and identified lipid species from 13 different classes (Fig 2A, B). The distribution of polar lipid classes in grouped and socially isolated mice appeared similar, with the largest percentage of polar lipids being attributed phosphatidylcholine (PC) (Fig. 2A). There was an absolute increase in the amount of polar lipids measured in socially isolated animals; however, this increase was not statistically significant (Fig. 2B). Among the 13 polar lipid classes profiled, we observed significant increases in ether-linked phosphatidylcholine (ePC), ether-linked phosphatidylserine (ePS), and lysophosphatidylcholine (LPC) (Fig.3). Interestingly, when polar lipid species were ranked by significant difference in isolated vs. grouped animals, LPC (16:0) was the highest ranked lipid and three other LPC species (18:0, 18:1 and 16:1) were among the top eleven lipids (Table. 1). These data identify significant changes to specific polar lipid species in socially isolated animals' mammary fat, in particular an increase in LPC lipids.

Increased neutral lipid classes in the mammary fat of socially isolated animals.

The primary roles of the adipocyte involve the storage and mobilization of lipids. Lipids stored within the adipocyte are packaged as neutral triglyceride species, which can be mobilized from the cell as free fatty-acids to provide energy metabolites to other cells and tissues. Additionally, stored neutral lipids can act as precursors to or constituents of other lipid-containing molecules, including polar lipids. To determine whether stored neutral lipids in social isolates mirrored the differences observed in their polar lipid species (i.e. an increase in 16 and 18 carbon, saturated and unsatured fatty acid chains), lipidomics profiling was performed on neutral lipid extracts from isolated vs. grouped mice.

Of the neutral lipids profiled, we observed significant increases that paralleled the increases in LPC species, with significant increases in 16:0, 16:1, 18:0, and 18:1 containing neutral lipids (Fig. 4, Table 2). We also observed a significant increase in 14:0 containing neutral lipids (Fig. 4, Table 2). Taken together, these data indicate that social isolation is associated with dramatic changes to neutral and polar lipid species in the mammary adipose tissue of socially isolated female mice.

LPC is toxic to TAg breast cancer cells in culture.

Lipidomics analysis identified LPC lipid species as being significantly increased in the mammary adipose tissue of socially isolated TAg mice. Therefore, we sought to determine the effects of exogenous LPC on a TAg cancer cell line derived from an invasive TAg mouse tumor (M6 cells,(9)). In the presence of 2.5% FBS, LPC doses of

100uM and below inhibited proliferation and increased cell death of M6 cells, whereas doses above 100uM resulted in the death of all cells in as little as 8hrs (Fig. 5). Under serum free condtions all LPC concentrations were toxic to the cells (Fig. 5).

LPC in the presence of mammary fat conditioned media promotes TAg breast cancer cell proliferation and protects from serum deprivation-induced cell death.

Typically, LPC is highly unstable in circulation, largely due to its rapid conversion to LPA by the actions of the serum phospholipase-D, autotaxin (10). LPA is an important signaling lipid that has been implicated in cancer cell motility, survival, and proliferation (10). We performed qPCR and Western analyses and did not observe the presence of autotaxin mRNA or protein in the M6 cells (data not shown); thus, we hypothesized that LPC toxicity results from the inability of M6 cells to convert LPC to LPA. Within the tumor microenvironment, mammary adipose tissue could serve as an abundant source of autotoxin (11), therefore we tested the hypothesis that LPC can be converted to LPA to inhibit LPC-mediated toxicity and potentially promote cancer cell proliferation and/or survival.

M6 cells were treated with low doses of LPC (5uM or 10uM) in serum free media or serum free media that had been conditioned with mammary fat from TAg animals. Under serum free conditions, 5uM LPC did not affect the survival or proliferation of the M6 cells; however, 10uM LPC resulted in a marked increase in cell death and decrease in proliferation (Fig. 6A, B). Interestingly, in the presence of mammary fat-conditioned media, LPC inhibited cell death in a dose dependent manner and resulted in slightly increased cell proliferation (Fig. 6C, D). These data suggest that LPC affects cancer cell apoptosis and proliferation after conversion to LPA and that mammary fat conditioned media contains the necessary enzyme for this conversion. To test for the presence of phospholipase-D activity in mammary fat conditioned media, bis-(p-nitrophenyl) phosphate (BNPP; a phospholipase-D substrate that is cleaved to p-nitrophenol) was added to conditioned media or serum free media and the conversion to p-nitrophenol (a yellow product) was determined using spectrophotometry. Following 8 hrs in culture, mammary fat conditioned media showed substantial accumulation of p-nitrophenol when compared to BNPP in serum free media alone (Fig. 7). Taken together, these data suggest that LPC can promote breast cancer cell proliferation and survival following exposure to mammary fat secreted phospholipase-D and conversion to LPA.

LPA promotes survival and proliferation of TAg breast cancer cells under serum free conditions.

The previous experiments revealed that mammary fat secreted enzymes are capable of converting LPC to LPA. Furthermore, LPC transitions from being toxic to TAg cells to being pro-tumorigenic when provided in the presence of mammary fat

conditioned media. These data suggest that LPA derived from LPC is a pro-tumorigenic factor to TAg cells. Therefore, we next determined whether exogenous LPA was sufficient to allow for cancer cell proliferation and/or promote survival under serum free conditions. M6 cells were cultured under serum free conditions +/- increasing doses of LPA. As expected, M6 cells without serum growth factors arrested proliferation and underwent substantial apoptosis (Fig. 8A). Conversely, cells treated with LPA displayed dose dependent increases in proliferation and inhibition of cell death (Fig. 8B). Taken together, these data suggest that LPC can inhibit cancer cell death and promote cancer cell proliferation acting as an intermediate metabolite that is converted to LPA by the actions of mammary fat secreted autotoxin.

Key Research Accomplishments

- We have identified that metabolic gene changes associated with social isolation and increased tumor burden in female mice occur within the mammary adipocytes.

- We have determined that the metabolic gene expression changes in mammary adipocytes and associated with social isolation are not dependent on the background mouse strain.

- We have observed that gene expression changes are specific to the adipocytes of the mammary gland and are not observed in other fat depots.

- The gene expression changes observed in mammary adipocytes of socially isolated animals correlate with functional metabolic changes including increased glucose consumption and increased lipid synthesis.

- In addition to metabolic changes in mammary adipocytes of socially isolated animals, we have observed elevated levels and secretion of Leptin protein in isolated vs. grouped animals' mammary adipose tissue.

- Mammary fat conditioned media from social isolates potentiates the proliferation of cancer cells compared to media made using grouped animal mammary fat, suggesting adipocyte secreted proteins/metabolites are linked to the increased tumor burden observed *in vivo*.

- The findings detailed above have been compiled into a manuscript that has recently been accepted for publication in *Cancer Prevention Research*.

- We have further characterized the metabolic gene expression changes within the mammary adipose tissue of social isolates and have identified upregulation of CHREBPα/β as potentially import transcription factors coordinating increased lipid synthesis within the mammary fat of social isolates.

- We have performed lipidomics profiling of lipids extracted from the mammary adipose tissue of isolated vs. grouped mice and have identified dramatic changes in several polar and neutral lipid species within social isolates.

- Lysophosphatidylcholine (LPC), the major precursor to the important signaling lipid, lysophosphatidic-acid (LPA), is significantly increased in social isolates' mammary fat pads.

- LPC inhibits proliferation of TAg cancer cells at low doses and is toxic to cancer cells at high doses. However, when TAg cancer cells are treated with LPC in the presence of mammary fat conditioned media, LPC is antiapoptotic and proliferative.

- Mammary fat conditioned media has phospholipase-D activity, thus can convert LPC to LPA.

- LPA protects SV40-TAg breast cancer cells from serum starvation-induced apoptosis and allows for proliferation in the absence of serum factors.

Reportable Outcomes

Presentations:

Chronic social stress in a mouse model of triple-negative breast cancer is associated with oncogenic changes in mammary fat. Volden PA, Wonder E, Skor M, Patel F, Conzen SD, Brady MJ. AACR 2013, April 6-10 in Washington, DC.

Mammary adipoctye-specific alteration are associated with paracrine effects on mammary tumoorigenesis. Volden PA, Wonder E, Brady MJ, McClintock MK, Conzen SD. AACR 2012, April 2-6 in Chicago, IL

Determining Fatty Acid Aliphatic Chain length by Isotropic Mixing Sachleben JR, Yi R, Volden PA, Conzen SD. Experimental NMR, April 2012 in Miami, FL

Environmental stress and breast cancer biology: What is the link? Conzen SD, Volden PA, Brady MJ, McClintock MK. AACR 2011: 102nd Annual Meeting, April 2-6 in Orlando, FL

Mammary adipocyte-specific metabolic alterations are associated with paracrine effects on mammary tumorigenesis. Paul A. Volden, Erin L. Wonder, Maxwell N. Skor, Christopher M. Carmean, Honggang Ye, Masha Kocherginsky, Eleanor Smith, Steven Kregel, Martha K. McClintock, Matthew J. Brady, and Suzanne D. Conzen. Keystone: Cancer metabolism, 2010, Banff, British Columbia

Environmental stress and breast cancer biology: What is the link? Conzen SD, Volden PA, Brady MJ, McClintock MK. AACR 2011: 102nd Annual Meeting, April 2-6 in Orlando, FL

Publications:

Chronic social isolation is associated with metabolic gene expression changes specific to mammary adipose tissue. Volden PA**,** Wonder EL, Skor MN, Carmean CM, Ye H, Kocherginsky M, Kregel S, McClintock MK, Brady MJ, Conzen SD. Cancer Prevention Research. In press

The Influence of Glucocorticoid Signaling on Tumor Progression. Volden PA**,** Conzen SD. Invited mini-review. Brain, Behavior, and Immunity. Brain Behav. Immun. 2013;30 Suppl:S26-31.

Conclusions:

Our previous work has identified an association between chronic stress, metabolic alterations within mammary adipocytes, and increased mammary tumor burden in female TAg mice (6). Because mammary fat conditioned media from socially-isolated vs. group-housed mice potentiates *in vivo* proliferation of TAg breast cancer cells, we hypothesized that mammary adipocyte-specific metabolic alterations in social isolates occur with concomitant pro-tumorigenic changes to proteins and/or metabolites secreted from the mammary adipocytes. Indeed, mammary adipose tissue from social isolates had elevated leptin protein levels and secreted more leptin when cultured *ex vivo* (appendix1, section 1, Fig. 4A, B, C). Interestingly, TAg cancer cells did not express the leptin receptor and, therefore, did not respond to exogenous leptin treatment. This result suggests that other factors secreted from the mammary fat may be involved in the increased tumor burden observed in social isolates.

Our recent investigations focusing on the metabolic alterations in mammary fat have identified LPC as a potential metabolite involved in the larger tumor formation within social isolates. Lipidomics profiling identified significant increases in the LPC lipid class within the mammary adipose tissue of social isolates, the most significant changes occurring in saturated and mono-unsaturated 18- and 16-carbon LPCs. In parallel, we observed increased neutral lipids containing saturated and mono-unsaturated 18- and 16-carbon acyl chains. Additionally, although LPC was toxic to TAg cancer cells in standard culture, we observed enhanced TAg cell proliferation and dose-dependent protection from apoptosis when TAg cells were treated with LPC in the presence of mammary fat conditioned media. An assay of phospholipase-D activity revealed that mammary fat conditioned media contains enzymatic activity predicted to convert LPC to the important signaling lipid, LPA. Similar to what was observed with LPC plus conditioned media, LPA treatment alone dose-dependently protected TAg cells from serum starvation-induced cell death and allowed for proliferation under serum free condtions. Taken together, these data identify LPC and LPA as potentially important lipids involved in social isolation-associated increased mammary tumor burden in TAg female mice.

Ongoing experiments have been designed to elucidate the roles of LPC and LPA within isolated vs. grouped animals, *in vivo.* Gene expression analyses suggest that TAg tumors express LPA receptors; however, we have not observed differences in receptor expression between grouped and isolated animals (data not shown). We will perform lipidomic analysis on serum from grouped and isolated animals to determine whether LPC or LPA differences can be detected in circulation. Furthermore, we will measure LPA and LPC levels in mammary fat-conditioned media from to determine whether LPCs or LPAs are differentially secreted from isolated vs. grouped animals. Further indication that LPA signaling contributes to the increased tumor burden in social

isolates will provide the rationale necessary for *in vivo* experiments aimed at antagonizing the LPC-autotaxin-LPA signaling axis. Indeed, small molecule inhibitors of autotaxin and LPA receptors are currently available and antagonizing autotaxin and/or LPA signaling has shown promise in pre-clinical cancer studies (10, 12).

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Appendices, section 1:

Chronic social isolation is associated with metabolic gene expression changes specific to mammary adipose tissue

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Abstract

Chronic social isolation is linked to increased mammary tumor growth in rodent models of breast cancer. In the C3(1)/SV40 T-antigen FVB/N (TAg) mouse model of "triple-negative" breast cancer, the heightened stress response elicited by social isolation has been associated with increased expression of metabolic genes in the mammary gland before invasive tumors develop (i.e. during the *in situ* carcinoma stage). To further understand the mechanisms underlying how accelerated mammary tumor growth is associated with social isolation, we separated the mammary gland adipose tissue from adjacent ductal epithelial cells and analyzed individual cell types for changes in metabolic gene expression. Specifically, increased expression of the key metabolic genes *Acaca, Hk2* and *Acly* was found in the adipocyte, rather than the epithelial fraction. Surprisingly, metabolic gene expression was not significantly increased in visceral adipose depots of socially isolated female mice. As expected, increased metabolic gene expression in the mammary adipocytes of socially isolated mice coincided with increased glucose metabolism, lipid synthesis, and leptin secretion from this adipose depot. Furthermore, application of media that had been cultured with isolated mouse mammary adipose tissue (conditioned media) resulted in increased proliferation of mammary cancer cells relative to group-housed conditioned media. These results suggest that exposure to a chronic stressor (social isolation) results in specific metabolic reprogramming in mammary gland adipocytes that in turn contributes to increased proliferation of adjacent pre-invasive malignant epithelial cells. Metabolites and/or tumor growth-promoting proteins secreted from adipose tissue could identify biomarkers and/or targets for preventive intervention in breast cancer.

Introduction

Human epidemiological studies have revealed that social isolation is associated with an increased risk of both all-cause mortality and metabolic diseases such as diabetes (1). Although association studies examining social isolation and human cancer risk have shown mixed results (2), the conclusions of these studies are likely inconsistent because human populations have immense genetic and environmental variation as well as heterogeneous breast cancer subtypes (3, 4) . These issues make identifying the mechanisms connecting social stressors to breast cancer biology challenging. They also underscore the importance of developing well-defined preclinical models for identifying the specific biological mechanisms linking an individual's response to social stressors to specific cancer subtypes.

Recent models of breast cancer examining the effects of imposed social isolation, a well-defined chronic stressor for female rodents, have found an association with increased mammary tumor growth. For example, in SV40 T-antigen FVB/N (TAg) mice (5) and Sprague-Dawley rats (6), social isolation was associated with larger mammary gland tumor burden and increased tumor invasiveness independently of changes in circulating estrogen and progesterone levels. Furthermore, our laboratories discovered that genes encoding key enzymes regulating lipid metabolism were differentially upregulated in the mammary glands of socially isolated versus group-housed mice, even prior to differences in tumor development (5). These results suggested that changes in lipid metabolism (in the pre-malignant epithelial cells and/or the adjacent adipocytes and stromal cells) could be driving the relatively aggressive mammary tumor growth of the social isolates.

In mouse models, social stressors have been linked to obesity (7), disruption of metabolism (8, 9), and diabetes (10), supporting an association between exposure to social stressors, the physiological stress response, and metabolic disorders. However, the mechanisms through which disrupted metabolism promote mammary tumorigenesis are still unclear. A link between metabolic syndrome/obesity and breast cancer has

been suggested (11). Obesity is associated with increased local production of estrogen in mammary gland fat likely contributing to estrogen receptor positive (ER+) breast cancer progression (12). However, emerging data also link metabolic diseases to ER negative (ER-) breast cancer, suggesting that factors other than estrogen are involved (13). Indeed, there is increasing evidence that in addition to estrogenic factors, mammary gland tumorigenesis can be influenced by both local and systemic metabolic signaling molecules, including insulin and leptin (14-16). Adding complexity, the stromal compartment and its reciprocal communication with the mammary epithelium is likely an important factor influencing breast cancer (17). Many cell types, including fibroblasts, adipocytes, and immune and endothelial cells compose the mammary stroma. Mammary gland adipocytes are arguably the least well-understood component. The relative lack of studies on mammary adipocytes in breast cancer biology is surprising considering the abundance of mammary gland adipose tissue and the well-established role of fat as an endocrine/paracrine tissue (18).

Endocrine action by adipose tissue includes the release of growth factors, hormones, and cytokines as well as adipocyte-specific factors (adipokines), many of which have been implicated in cancer progression (19). For example, leptin, an adipokine that was first identified as a gene product influencing satiety and body mass (20), has since been shown to affect the differentiation and proliferation of other cell types, including breast cancer cells (21). Adding to the complexity of adipocyte endocrine action is the fact that the metabolic activity and the profile of secreted substances in adipose tissue varies depending on its location (e.g. visceral vs. subcutaneous depots) (22). Properties specific to mammary adipose tissue and the mammary microenvironment that influence breast cancer biology remain largely unexplored. Therefore, whether abnormal function in mammary adipocytes and the ensuing effects on local metabolism contribute to estrogen receptor (ER)-independent breast cancer biology has not been established.

Using a global gene expression approach, we previously identified key metabolic genes including acetyl-CoA carboxylase alpha *(Acaca)*, hexokinase 2 *(Hk2)*, and ATP

citrate lyase *(Acly)* as significantly overexpressed in the mammary glands of isolated versus group-housed TAg mice. Interestingly, increased expression of these gene products is associated with the hallmark metabolic changes observed in cancer cells (23). However, in our previous experiments RNA was obtained from whole mammary gland tissue so we could not determine the specific cell type(s) that were contributing to increased metabolic gene expression. Because it has become increasingly clear that mammary epithelial cell proliferation is influenced by adjacent non-epithelial stromal cells (17), we sought to establish the specific cell types contributing to mammary gland metabolic gene expression changes. Our new findings reveal an association between social isolation, the ensuing stress response, and increased mammary gland adipose tissue lipid metabolism, without a measurable concomitant effect on systemic metabolism. While previous studies have implicated increased mammary fat estrogen production in ER+ breast cancers, our results implicate mammary adipocyte function and its secretome as an important modulator in a model of ER-negative breast cancer growth.

Materials and Methods

C3(1)/SV40 TAg FVB/N transgenic mice and CD1 outbred mice

FVB/N mice homozygous for the SV40 *TAg* transgene (originally provided as hemizygous TAg mice by Jeff Green of the National Cancer Institute's Mouse Models of Cancer Consortium), non-transgenic FVB/N mice (Charles River) and Swiss CD1 mice (Charles River) were weaned at 3 weeks of age and transferred to differential housing as described in the Supplementary Methods section. TAg homozygous animals were maintained and bred to generate TAg homozygous study populations. Female TAg mice were no longer bred following birth of a litter. To minimize confounding influences from estrous cycle hormones on experimental results, all study animals were sacrificed in estrus phase, as determined by vaginal cytology (24). National Institutes of Health and University of Chicago Animal Care Guidelines were followed for all studies. A detailed

outline of experiments and setup prior to animal sacrifice are described in supplemental methods.

Measurement of circulating factors and food/caloric consumption

Blood glucose was measured via tail bleed using a Bayer contour glucose meter. Tail blood was collected for plasma isolation using heparinized capillary microvettes (Andwin Scientific) and was diluted for corticosterone measurements 1:50 in buffer provided with a corticosterone ELISA kit (Enzo Life Sciences). Immediately at sacrifice, cardiac puncture was performed to collect blood, and serum was isolated and stored at -80^{O} C. Serum insulin and leptin were measured by ELISA (ALPCO Diagnostics, Crystal Chem.; respectively). Serum free-fatty acids were measured by enzymatic assay (Wako Diagnostics). Food consumed was calculated weekly as initial food mass minus the final mass at week's end. Calories consumed were calculated by multiplying the consumed food mass by the diet's caloric density (Teklad #8904, 3.0kcal/g). Statistical analyses are provided in supplemental methods.

Adipose tissue harvest, collagenase digestion, and centrifugal separation of mammary adipocytes

Mice were sacrificed at 15 weeks of age and mammary fat pads with palpable tumors were excluded from experiments. Gonadal fat pads were excised from their adjacent fallopian tubes and snap-frozen. Excised mammary fat pads were immediately placed in 2 mL microcentrifuge tubes containing 700 μL DMEM with 10% FBS. Details of adipose tissue harvest and adipocyte isolation are provided in supplementary methods and are a modification of the procedure reported previously (25).

Quantitative RT-PCR

mRNA (1ug) was reverse-transcribed using the qScript cDNA synthesis kit (Quanta Biosciences). Q-RT-PCR was performed with PerfeCTa SYBR Green FastMix (Quanta Biosciences). All reactions were performed in a Biorad iCycler iQ real-time

PCR system. Details of the statistical analysis are provided in supplemental methods section.

Adipocyte glucose consumption and lipogenesis

For each 15 week old animal, mammary fat pads were minced in 1 mL of DMEM with 1% BSA. Following mincing, 4 mL of 1.0% BSA and 2 mg/mL collagenase (Type II, Worthington) were added. Tubes were agitated at 37◦C for 60 min, and pipette-mixed every 15 min. Cells were filtered through a 100 µm nylon mesh and then spun at 100 X *g* for 60 seconds. Floating adipocytes were transferred to microcentrifuge tubes and repeat centrifugation (30 sec, 100 X *g*) followed by media removal with syringe and needle were performed to obtain isolated packed adipocytes.

To compare adipocyte glucose consumption, adipocytes (10 µL) from individual animals (group-housed n=5; socially isolated n=3) were placed into 96 well plates containing 90 µL of DMEM (1 g/L glucose) and 1% BSA. Cells were stimulated with 50 nM insulin or vehicle control, incubated for 4 h, and medium was then collected and glucose consumption measured as loss of glucose from the media between 0 and 4 h (26). Statistical analyses are provided in supplemental methods.

To assess lipogenesis, 10 µL of adipocytes from individual animals (n=3, each condition) were placed into scintillation vials containing 400 µL of DMEM (1 g/L glucose) and 1% BSA. Cells were treated with vehicle or 10 nM insulin and 100 µL of ^{14}C-labeled glucose (0.001uCi/mL, 1uCi /Rxn) was immediately added to each vial. Cells were incubated at 37^{O}C for 60 min and 4 mL of Betafluor was added, followed by vortexing. The following day, 3.2 mL of the upper (lipid-containing) fraction was transferred to a new scintillation vial and radioactivity measured using a scintillation counter. Statistical analyses are provided in supplemental methods.

Leptin measurements

Mammary adipocytes were sonicated in 1X PBS containing protease inhibitors, and spun at 4^{O}C and 13,400 X *g* for 15 min. The infranatant between the top lipid layer

and pelleted nuclei that contained soluble proteins was transferred to a new tube. For Western blot analysis, 20 µL of mammary adipocyte protein lysate from individual animals (n= 5 per housing condition) was boiled with 4X Laemmli buffer for 10 min. Samples were resolved on a 15% SDS gel, transferred to PVDF (Millipore), and immunoblotted with anti-leptin antibody (A-20, Santa Cruz Biotechnology) while anti-cyclophilin-B antibody was used as a loading control (PAI-027, Affinity Bio-Reagents). Leptin ELISA (Crystal Chem Inc.) assays were performed on protein lysates (group-housed, n=7; socially isolated, n=5), following BCA-based protein normalization (Thermo Scientific). Leptin receptor immunoblotting was performed with Abcam 5593 (Supp. Fig. 3B). Statistical analyses methods are provided in supplemental methods.

For secreted leptin measurements, fat pads from 4 grouped or 4 socially isolated animals were pooled, minced and spun at 100 X *g* for 1 min. The floating tissue was weighed and 1 g incubated with 10 mL of media (SH30240.01, Hyclone), containing 1% BSA and 1% penicillin/streptomycin (P/S), at 37^{O}C for 24 h. Medium was harvested and sterile-filtered through a 0.22 µm syringe filter, aliquoted, and stored at -80^{O}C. Media (40ul) was used for ELISA leptin measurements (Crystal Chem Inc.). Statistical analyses are provided in supplemental methods.

Pre-malignant SV40-TAg-mammary epithelial cell proliferation

The SV40-Tag M27H4 mammary cell line (a kind gift of Dr. Cheryl Jurcyk, Boise State University) was originally isolated from a hyperplastic, non-invasive lesion in a 2 month old C3(1)/Tag mouse (27). The cells were authenticated by microscopic morphology and were routinely tested via growth curve comparison to the original series of cell lines (27). Cells (5000/well, ~50% confluence after 16hrs) were seeded in 96-well plates containing DMEM, 10% FBS, and 1% P/S. After 16 hrs, media was removed and cells were washed with PBS and then cultured with either 100 µL of mammary adipose tissue culture media from grouped or isolated animals (described above), or in control media (serum free, 1% BSA, 1% P/S). Replicate wells from each condition were washed with PBS and fixed with 10% TCA at 24 hour intervals. On the final day, total protein from

each well, representing relative cell number, was measured via the SRB method (28). The SRB assay was also used to measure proliferation of M27H4 cells in response to recombinant leptin (Life Technologies). Statistical analyses are provided in supplemental methods.

Results

Social isolation versus group housing is associated with increased vigilance followed by accelerated mammary tumor growth

Hemizygous FVB/N TAg female mice typically progress to palpable carcinomas at approximately 16 weeks of age (29), whereas homozygous mice can develop palpable mammary tumors as early as 10 weeks of age (30). Our previous work demonstrated that socially isolated homozygous TAg mice have a larger invasive tumor burden (the total palpable tumor volume per mouse) compared to group-housed mice (5). These results needed to be confirmed to investigate the metabolic and molecular changes underlying the increased tumor burden.

In agreement with our previous work (5), among the mice that had palpable tumors by 18 weeks of age (Supp. Fig 1A), isolated mice developed a significantly larger average tumor burden than group-housed mice (*$p=0.013$, Supp. Fig.1B). Also, we again observed that socially isolated mice had become more vigilant by 16 weeks of age, as demonstrated by a longer time to leave their home base and explore a novel environment ($p<0.0001$; log-rank test, Supp. Fig.1C). These results confirm that exposure to social isolation is associated with increased vigilance and increased mammary tumor growth (5).

Social isolation is associated with metabolic gene expression changes in mammary adipocytes

Next, we sought to determine whether the increased tumor burden of socially isolated mice was associated with local and/or systemic changes in lipid metabolism.

Our previous studies in the TAg model found that social isolation results in increased mRNA steady-state levels of key genes encoding glucose metabolism and lipid synthesis enzymes (*Hk2, Acaca*, and *Acly)* in whole mammary gland extracts from 15 week old mice, before differences in tumor formation (5). Increased glucose metabolism and *de novo* lipid synthesis are associated with the hallmark metabolic changes observed in cancer cells. However, *de novo* synthesis of lipids from glucose is also a primary function of adipocytes, and therefore one of or both of these cell populations could account for the observed overall change in mammary gland metabolic gene expression. To separate the cell types, we used a collagenase digestion and centrifugation protocol (25) and isolated the floating adipocytes from other cells in minced mammary glands of 15-week old mice. Representative images of fractionated cells are shown in Figure 1A. mRNA was isolated from each fraction and gene expression was analyzed by Q-RT-PCR.

A comparison of the relative gene expression in the adipocyte versus stromal/epithelial fraction (regardless of housing condition) revealed 20-60 fold higher overall metabolic gene expression in the adipocytes (Fig. 1B). Adipocytes from socially isolated TAg mouse mammary glands expressed significantly higher steady-state *Hk2* ($2^{-\Delta\Delta Ct}$=1.84, *p=0.012), *Acly* ($2^{-\Delta\Delta Ct}$=2.93, ***p=0.0001) and *Acaca* ($2^{-\Delta\Delta Ct}$=2.94, ***p=0.0001) mRNA levels compared to mRNA from group-housed mammary gland adipocytes (Fig. 1C). In cells of the non-adipocyte fraction, expression of these genes was not statistically different between isolated and group-housed animals (p>0.12 for all three genes, Fig 1D, Supp. Table 1). These results indicate that these metabolic gene expression changes in the mammary gland following social isolation occur primarily in the adipocyte tissue fraction.

To determine whether the elevated metabolic gene expression was specific to the mammary gland fat depot or similarly found in other adipose tissue depots, we also harvested gonadal fat attached to the uterus and fallopian tubes. Unlike mammary adipose tissue, there were no significant differences in the gonadal fat metabolic gene expression from the isolated versus grouped mice (Fig. 1E, Supp. Table 1, p>0.43 for all

three genes), suggesting that the mammary adipocytes were more sensitive to stress-induced upregulation of metabolic gene expression.

Social isolation is not associated with detectable systemic metabolic changes

Our gene expression data suggested the intriguing possibility that social isolation in female rodents is associated with mammary adipose-specific metabolic changes; therefore, we performed additional analyses of systemic metabolism to examine the local versus systemic effects of social isolation on metabolism. We measured food consumption and weight in isolated and grouped cohorts. Animal weights did not differ between isolated and group-housed mice prior to palpable tumor formation (age 10 wks, Table 1) or after tumor formation (age 17 wks, Table 1). However, isolated mice consumed significantly more kilocalories per day compared to group-housed mice both before palpable tumor formation (age 8-10 wks; $p=0.03$, Table 1) and after (age 11-17 wks; $p=0.0016$, Table 1), suggesting a possible effect of social condition on eating behavior and/or systemic energy metabolism.

To test this possibility, parallel cohorts of chronically isolated and group-housed female TAg mice were placed in individual metabolic cages. This was also a test of the enduring effects of living in groups, as all mice had to be isolated during the metabolic cage studies because grouped metabolic cages are not available. When animals were placed in the individual metabolic cages, we did not detect any systemic metabolic differences between the previously grouped and isolated cohorts (Supp. Fig.2 and Supp. Table 2). In addition, the previously observed differences in food consumption (Table 1) were no longer evident ($p=0.80$ active, $p=0.27$ inactive period, Supp. Table 2), suggesting that the superimposed stress of social isolation in the metabolic cages affected the eating behavior of the grouped mice.

In addition to food consumption, animal weight, and metabolic cage analyses, we measured several circulating markers of systemic metabolism while the animals were in their assigned social conditions. As shown in Table 1, at 15 wks of age, we did not observe significant differences in systemic circulating blood glucose, serum insulin,

serum free-fatty acids, or serum leptin, even though profound changes were seen in mammary adipose gene expression at this age. Thus, circulating metabolic factor levels did not suggest a significant effect of social isolation on systemic metabolism.

Upregulation of metabolic genes in the mammary gland occurs independently of the SV40 TAg transgene and is not limited to the FVB/N mouse strain

The TAg transgenic mouse breast cancer model used in this study is on the FVB/N background strain. In addition, previous studies have shown that different mouse breast cancer models (e.g. *MMTV-neu* vs. *MMTV-pymt)* on the FVB/N background strain can have differential effects on metabolic phenotypes including obesity (31). To rule out potential FVB/N-specific or SV40-TAg-associated effects of social isolation on mammary fat gene expression or systemic metabolism, we repeated the isolation versus group-housed studies using non-transgenic FVB/N (WT) and outbred CD1 female mice.

We measured whole mammary gland gene expression from chronically isolated and group-housed 15-wk-old female WT and CD1 mice. The results recapitulated the upregulation of *Hk2* (WT, *p=0.02; CD1, p=0.09), *Acly* (WT, ***p<0.001; CD1, **p=0.007), and *Acaca* (WT, *p=0.03; CD1, *p=0.01) steady-state mRNA that we observed in mammary glands from isolated 15-wk old TAg mice (Fig. 2A, B; Supp. Table 3). Moreover, as we had observed in the TAg mice, gene expression in gonadal fat was not significantly different between isolated versus group-housed female CD1 mice (Fig. 2C, p>0.59 for all genes), and only one of the three metabolic genes was significantly upregulated in the WT visceral fat (*Acaca* *p=0.04, *Hk2* p=0.47, *Acly* p=0.10; Fig. 2D).

As was observed in TAg mice, CD1 mice metabolic cage measures (Supp. Fig.2; Supp. Table 2) and other markers of systemic metabolism (Supp. Table 4) were not significantly different between grouped and isolated CD1 mice. Together, these results support the hypothesis that depot-specific upregulation of metabolic gene expression in

mammary fat is a broader characteristic of mammary fat from chronically isolated mice, rather than dependent on mammary tumor formation.

Upregulation of metabolic genes in mammary adipocytes results in their elevated glucose consumption and lipid synthesis.

Changes to cellular metabolism can be achieved through the increased expression of glucose metabolism and lipid synthesis gene products, such as the proteins encoded by *Hk2, Acly, and Acaca.* Phosphorylation of glucose by hexokinase 2 (encoded by *Hk2*) effectively traps glucose within cells for subsequent metabolic processes, including lipid synthesis (lipogenesis). *Acly* and *Acaca* gene products also play essential roles in regulating lipid synthesis pathways (Fig. 3A). Therefore, we sought to determine whether the upregulation of *Hk2*, *Acly*, and *Acaca* in the mammary adipocytes of socially isolated animals was associated with the predicted functional increases in glucose metabolism and/or lipid synthesis.

Following 15 wks of either group housing or social isolation, we purified mammary adipocytes from individual TAg mouse mammary glands and measured their relative cellular glucose uptake from culture media in the presence (stimulated) or absence (basal) of insulin. Under basal and stimulated conditions, mammary adipocytes from socially isolated animals consumed roughly twice the amount of glucose compared to adipocytes from group-housed animals (Fig. 3B; *p=0.025, Wilcoxon rank sum test).

In a parallel experiment, we assessed the relative amount of radio-labeled glucose incorporated into lipid (a measurement of lipogenesis) in mammary adipocytes from the same set of animals. Under basal conditions, lipogenesis was nearly twice as high in mammary adipocytes from socially isolated mice (Fig. 3C; *p=0.05). Following insulin stimulation, the level of *de novo* lipid synthesis in isolated animals' mammary adipocytes further increased to roughly 3-fold higher than group-housed animals' adipocytes (Fig. 3C; *p=0.05, Wilcoxon rank sum test). Taken together, these results demonstrate that increased *Hk2*, *Acly*, and *Acaca* mRNA expression in mammary adipocytes of socially isolated animals is indeed associated with a concomitant relative

increase in both glucose consumption and glucose incorporation into newly synthesized lipids.

Leptin expression and secretion is increased in adipocytes from socially isolated animals.

Both adipocyte glucose metabolism and lipid synthesis are suspected to be important regulators of production and secretion of the adipokine, leptin (32, 33). Furthermore, previous *in vitro* and *in vivo* studies have implicated leptin in cancer cell proliferation and tumor growth (34-37). Although there were no significant differences in circulating leptin between isolated and group-housed animals (Table 1), the metabolic changes we saw in the mammary adipose tissue of socially isolated mice suggested that adipocytes may increase leptin secretion within the local mammary microenvironment. Therefore, we measured the leptin content of mammary adipocytes from socially isolated vs. group-housed mice.

Using both Western blot and ELISA analyses, we observed approximately 60-70% more leptin in the mammary adipocytes of social isolates (Fig. 4A, B; ELISA, *p=0.01; Western blot, **p=0.009). To determine whether the elevation in intracellular leptin correlated with increased leptin secretion, we cultured mammary adipose tissue from isolated and group-housed animals for 24 hours under serum-free conditions. The media was then harvested and its leptin content was assessed. As observed in Fig. 4C, leptin levels were elevated in the mammary adipose tissue culture media from the socially isolated animals relative to media from group-housed mouse mammary adipose tissue (*p=0.02). Thus, social isolation and the ensuing stress response appear to result in increased mammary adipocyte leptin protein expression and secretion. In contrast, systemic levels were not significantly affected (Table 1), again indicating an effect of social isolation and associated neuroendocrine responses to stressors on gene expression specifically in the mammary adipose tissue microenvironment, as opposed to a generalized effect on all fat depots.

Mammary adipose tissue conditioned media potentiates the proliferation of SV40-TAg mammary epithelial cancer cells *in vitro*

Adipose tissue depots are now considered to be endocrine organs, secreting numerous factors including leptin. Because we observed elevated leptin secretion from the mammary fat of socially isolated animals, we hypothesized that differential secretion of leptin and/or other adipokine factors could contribute to the larger mammary tumor burden associated with social isolation. We next evaluated the possibility that secreted factors from mammary fat contribute to cancer cell proliferation, using conditioned media from the leptin secretion experiments (Fig. 4C).

Conditioned media was applied to an SV40 TAg transgenic mammary epithelial cell line (M27H4) derived from a *in situ* hyperplastic lesion (27). Media derived from culturing the mammary adipose tissue, regardless of animal housing, was sufficient to drive cancer cell growth without serum (Fig. 4D), indicating that secreted factors and/or metabolites from mammary adipose tissue can support cancer cell proliferation. Interestingly, media derived from culturing the mammary adipose tissue from socially isolated animals resulted in significantly more M27H4 cell proliferation than media from group-housed mouse mammary adipose tissue (***$p<0.0001$). However, addition of exogenous recombinant purified leptin (final concentration 0.1ng/mL-10ug/mL) did not affect cell proliferation (Supp. Fig. 3A), suggesting that factor(s) other than leptin drive proliferation of the epithelial cells in culture and may also contribute to the increased tumor burden observed in socially isolated animals. Thus, isolated animals' mammary adipose tissue appears to be enriched in the production of proliferative factors. Furthermore, differences in the secretome components (including leptin) likely contribute to the larger tumor growth seen in socially isolated versus group-housed mice.

Discussion

Although association studies examining social stressors and human cancer risk have shown mixed results, several rodent models suggest that the social stress

response can contribute to cancer progression (5, 6, 38-40). Because the biology of the stress response involves complex changes in physiology, identifying the precise aspects of the stress response that influence cancer biology is challenging. Here, we identify a relationship between exposure to a chronic social stressor, altered mammary adipose tissue metabolism, and breast cancer progression. Our results revealing metabolic alterations within the adipocytes of the mammary gland microenvironment expand the importance of maintaining metabolic homeostasis in cancer prevention.

It is well-established that exposure to unmitigating low-level psychosocial stressors is correlated with an increased metabolic and cardiovascular disease risk (41, 42). Social isolation, an established psychosocial stressor for female rodents, is associated with an increased corticosterone responsiveness to a mild acute stressor (e.g. restraint for 30 minutes) and with increased mammary tumor growth in both the TAg mouse (5) and Sprague-Dawley rat (6) models of breast cancer. Additional studies have also reported effects of psychosocial stressors in rodent models of mammary tumorigenesis (39, 40). Using a carcinogen-induced mouse breast cancer model, Boyd et al. observed increased expression of ER-alpha and promotion of mammary tumorigenesis in adulthood when neonates were exposed to chronic, moderate psychosocial stress (39). In a more general genetic mouse model of human cancer (p53 +/- mice), Hasen et al. reported an initial higher mortality rate in isolated female mice. Among the various types of cancers that arose in this model (p53 +/-), mammary cancer incidence was actually lower in the surviving social isolates, although other cancers appeared earlier and were higher in incidence in the isolates (40). Taken together these studies support a psychosocial influence on breast cancer biology.

Adipose tissue secretes numerous hormones, growth factors and adipokines, some of which have been linked to both inflammation (43, 44) and cancer progression (45-48). Of note, we observed elevated leptin secretion from the mammary fat of socially isolated animals. Despite this observation, the mouse mammary epithelial cancer cell line used in this study did not proliferate more when exposed to recombinant mouse leptin (Supp. Fig. 3A). This is likely because the mouse cell line does not

express detectable levels of the leptin receptor long isoform (Supp. Fig. 3B) required for full activity (49). However, expression of the leptin receptor long isoform was observed in tumor samples from TAg mice (Supp. Fig.3B). Thus, we cannot rule out an increase in the local mammary leptin concentration playing an important role in the increased tumor burden observed *in vivo* in socially isolated animals. Indeed, several studies support a role for increased leptin in breast cancer risk (14, 36).

The M27H4 cell line we used in this study was chosen because of its derivation from a TAg mouse mammary *carcinoma in situ*. However, mouse immortalized cells in culture inevitably undergo genotypic and phenotypic changes over time. We are currently attempting to identify the additional factor(s) in the conditioned media from adipose tissue that contribute to the differential effects on epithelial cell proliferation, and in the future will test the requirement for these factors on a variety of mammary epithelial cell lines and for their presence *in vivo.*

The finding that changes in metabolic gene expression are more prominent in mammary versus visceral fat depots was completely unanticipated. However, given the critical role of lactation in reproductive fitness, heightened transcriptional responsiveness of the mammary fat to stress hormones may allow preservation of milk production during times of chronic environmental stress exposure (50). In fact, the depot-specificity of adipose tissue adipokine secretion and hormone responsiveness is increasingly appreciated (51). Our observations that mammary adipose tissue displays a unique physiological association with chronic environmental stress exposure is in line with the current appreciation for ‘depot-specificity’ in adipose tissue biology (51). Furthermore, interactions between adipocytes and cancer epithelium have been increasingly reported in the literature (52); however, mammary fat as an independent depot and its specific influences on breast cancer biology remain largely uncharacterized. Our data raise important questions about local crosstalk between mammary adipocytes and epithelium in the *in situ* stages of breast cancer, when preventive intervention may be most relevant. For example, do metabolic changes in mammary adipocytes, independent of obesity, contribute to tumor incidence and

progression? Can mammary adipocyte biology be altered through a dietary intervention (e.g. increased omega-3 fatty acids) or with small molecules (e.g. metformin) to reduce secretion of pro-tumorigenic factors and cancer progression? Answers to these and other questions will require in-depth studies of mammary fat biology and biochemistry.

The exact neuroendocrine mechanisms by which the response to chronic social stressors triggers pro-oncogenic changes in mammary adipocyte physiology remain to be detailed. Notably, social isolation of female rodents is also associated with heightened systemic glucocorticoid responsiveness to superimposed stressors. Human adipose tissue responds to either chronically high or pulsed glucocorticoid exposure, which can in turn result in adipose depot-specific effects including fat redistribution, decreased insulin sensitivity and increased fatty acid efflux (53). *In vivo*, glucocorticoids may promote tumor growth and previous work suggests a predominant role for glucocorticoids and GR activation in inhibiting epithelial cell apoptosis (54). Based on our findings, we propose a model wherein social isolation and its ensuing neuroendocrine effects potentiate tumor growth by altering mammary adipocyte metabolism and associated metabolite/adipokine secretion, as well as by directly affecting mammary cancer cell apoptosis and possibly proliferation (Fig.5). Although much emphasis has been placed on the role of excess adiposity and its contribution to ER+ breast cancer (12), our findings suggest that local mammary adipocyte biology also influences ER-negative breast cancer.

Understanding the mammary adipocyte physiology associated with *in situ* and invasive tumor formation may aid in identifying new biomarkers and/or targets for breast cancer prevention and treatment. Furthermore, employing behavioral interventions and bolstering the social support of at-risk individuals, in addition to the obvious quality of life benefits, may have important physiological consequences relevant to cancer prevention. Our findings suggest that lifestyle and pharmacologic interventions (e.g. diet, exercise, and possibly, metformin) targeting metabolic abnormalities in adipose tissue may be effective preventive measures because of their ability to alter the local microenvironment that supports breast cancer development.

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Figure Legends

Fig. 1. Mammary gland cell fractionation followed by Q-RT-PCR measurement of gene expression from isolated and group-housed mice. Collagenase treatment followed by centrifugation was used to separate SV40 TAg mammary gland adipocytes (left panel) from the non-adipocytes (epithelial/stromal cells, right panel) (A). Relative *Hk2, Acly,* and *Acaca* gene expression in the adipocytes (white bars) vs. non-adipocyte (black) cells (B). Expression of *Hk2, Acly,* and *Acaca* normalized to beta-actin (*Actb*) mRNA expression in mammary adipocytes (C), non-adipocytes (epithelial/stromal cells) (D), and in visceral fat (E) in isolated (white bars) vs. grouped mice (black). Error bars indicate standard deviation. In panels C-E; *p<0.05, ***p<0.001. N = 4 and 7 for grouped and isolated animals, respectively.

Fig. 2. Relative mammary gland and visceral fat depot gene expression in differentially housed WT and CD1 mice**.** *Hk2, Acly,* and *Acaca* gene expression from 15 week old CD1 mammary glands (A; n=4 grouped, 5 isolated) or WT mammary glands (B; n=11 grouped, 9 isolated). *Hk2, Acly,* and *Acaca* gene expression at 15 weeks in CD1 mice gonadal adipose tissue (C; n=3 grouped, 4 isolated) or in WT mice gonadal adipose tissue (D; n=12 grouped, 9 isolated). Socially Isolated mRNA expression (white bars) relative to group-housed mRNA expression (black). Error bars indicate standard deviation. In all panels; *p<0.05, ***p<0.001, NS=not significant.

Fig. 3. Glucose consumption and lipogenesis in isolated mammary adipocytes from differentially housed TAg mice**.** A, *Hk2, Acly,* and *Acaca* gene products involved in glucose and lipid metabolism. B, mammary adipocytes from group housed (n=5) and socially isolated (n=3) animals were cultured in low glucose media, +/- insulin, for 4 hours. Media was then harvested and glucose in the media was measured via the glucose oxidase method and consumption assessed by a comparison to glucose in fresh (not used for culture) media. C, mammary adipocytes were purified from group housed and socially isolated animals (n=3 per group) and immediately cultured with ^{14}C glucose, +/- insulin, for 1hr. The lipid fraction was extracted from the adipocytes and

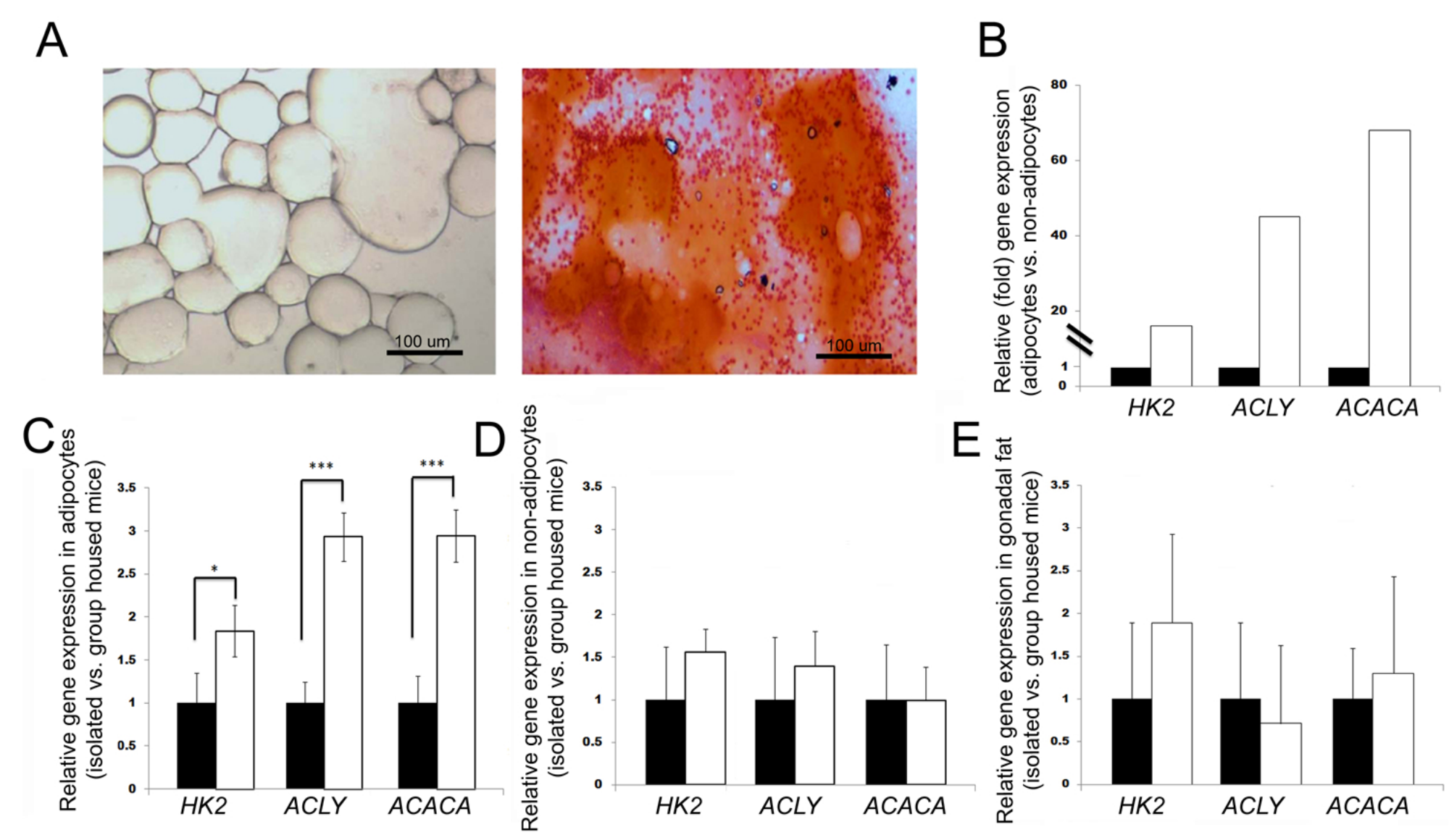
^{14}C, representing the incorporation of labeled glucose into lipid species, was counted using a scintillation counter. Average basal lipogenesis and glucose consumption values for grouped animals were normalized to 1. All other measurements are reported as relative to this value**.** $p \leq 0.05$, Wilcoxon rank sum test.

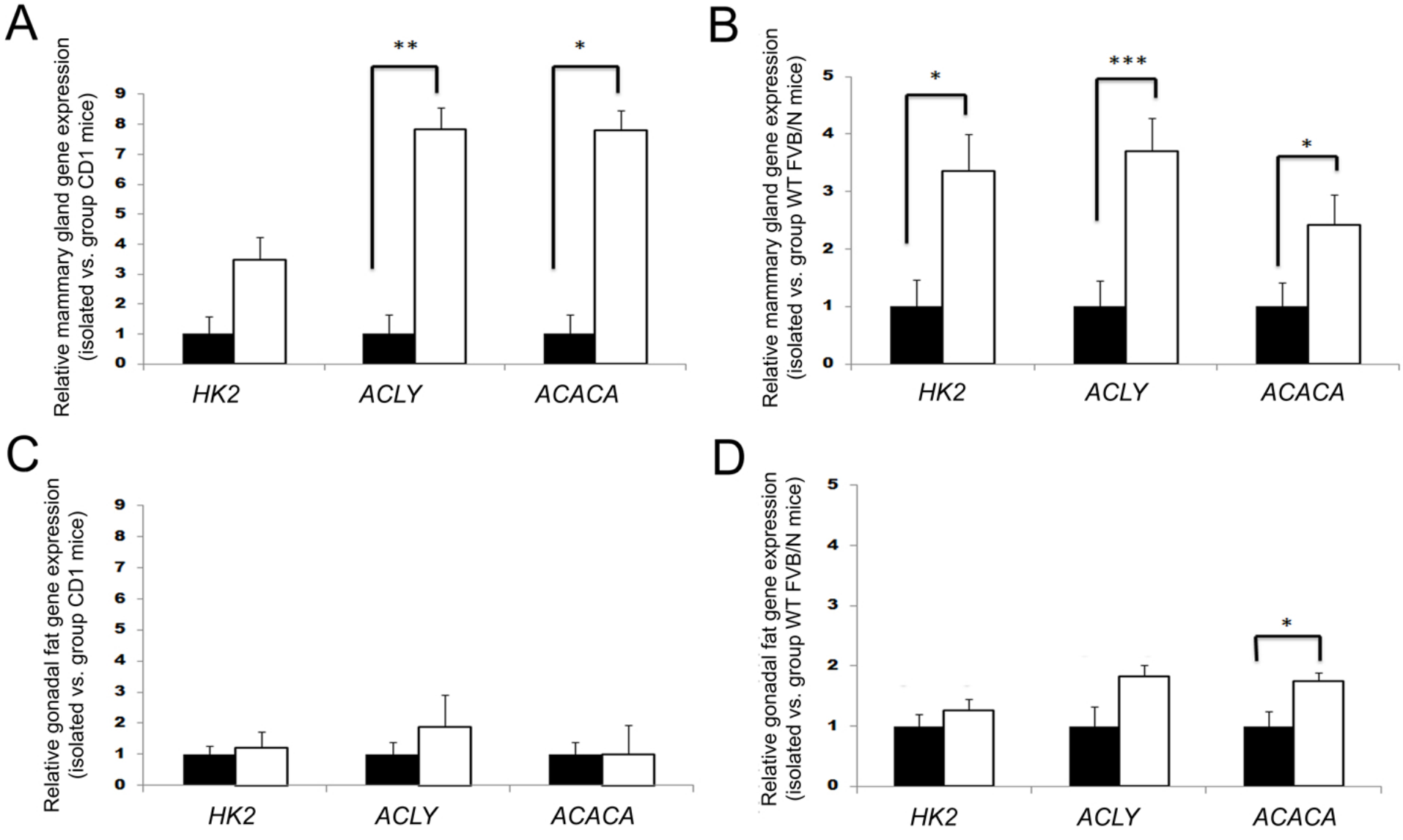
Fig. 4. Analysis of mammary adipose tissue lysates and secreted factors from differentially housed TAg mice. A, Representative Western blot showing grouped and socially isolated mouse mammary adipocyte lysates probed with antibodies targeting leptin, adiponectin, and actin proteins on the same blot. B, Relative leptin expression in mammary adipocyte lysates as measured by Western blot densitometry (n=5 per group) and ELISA (n=7 grouped, 5 isolated). C, Mammary adipocyte-secreted leptin from a 24hr adipocyte culture media, measured by ELISA (n=4 per group). Error bars indicate standard deviation; *p<0.05, **p<0.01, Wilcoxon rank sum test. D, Serum-free media incubated with isolated and group-housed mammary adipose tissue applied to the M27H4 SV40-TAg mammary epithelial cell line followed by measurement of cellular protein over time (SRB assay) reflecting total cell number. Serum-free DMEM with 1% BSA was used as a control. ***p<0.0001 isolated vs. grouped, error bars indicate standard deviation.

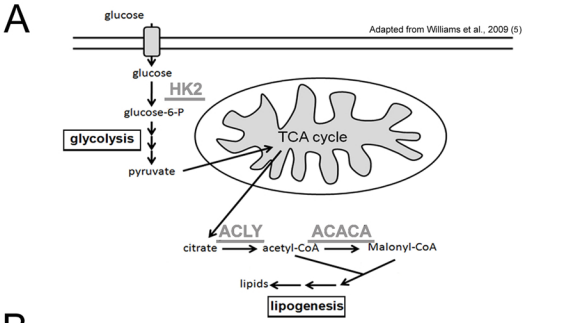
Fig. 5. Working model showing interactions between the social environment (exposure to a chronic stressor), the individual's ensuing stress response, and the neuroendocrine axis with mammary tumorigenesis. The HPA axis and sympathetic nervous system mediate the physiological response to chronic stressors such as social isolation. Altered neuroendocrine signaling in the mammary microenvironment results in metabolic changes within mammary adipocytes. These include changes in the mammary adipocyte secretome (e.g. increased leptin production) which can promote cancer cell proliferation. Neuroendocrine signaling (e.g. via glucocorticoids) could also act directly on the mammary epithelium and initiate oncogenic pathways.

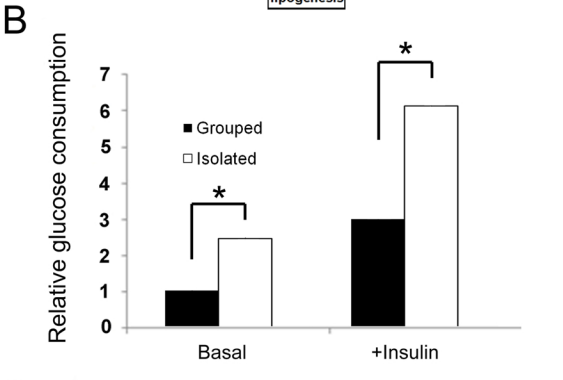
Table 1. Measurements of circulating metabolic parameters, food consumption, and weights in grouped vs. isolated TAg female mice. Circulating markers were obtained

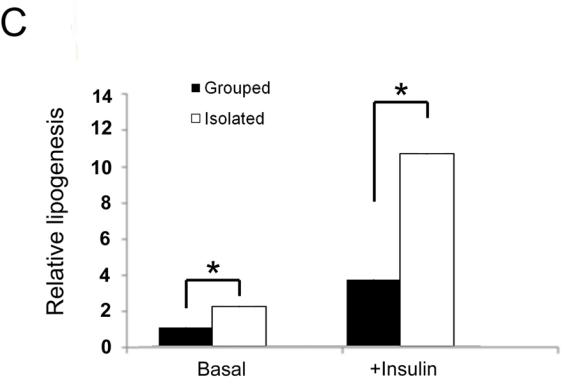
from 15wk old animals (9 group-housed, 8 isolated). Food consumption and weights (16 group-housed, 15 isolated) are divided into pre- and post-tumor time periods to investigate the effects of tumor burden on metabolism. Data indicate means +/- standards deviation, with p-value obtained using a student's T-test.

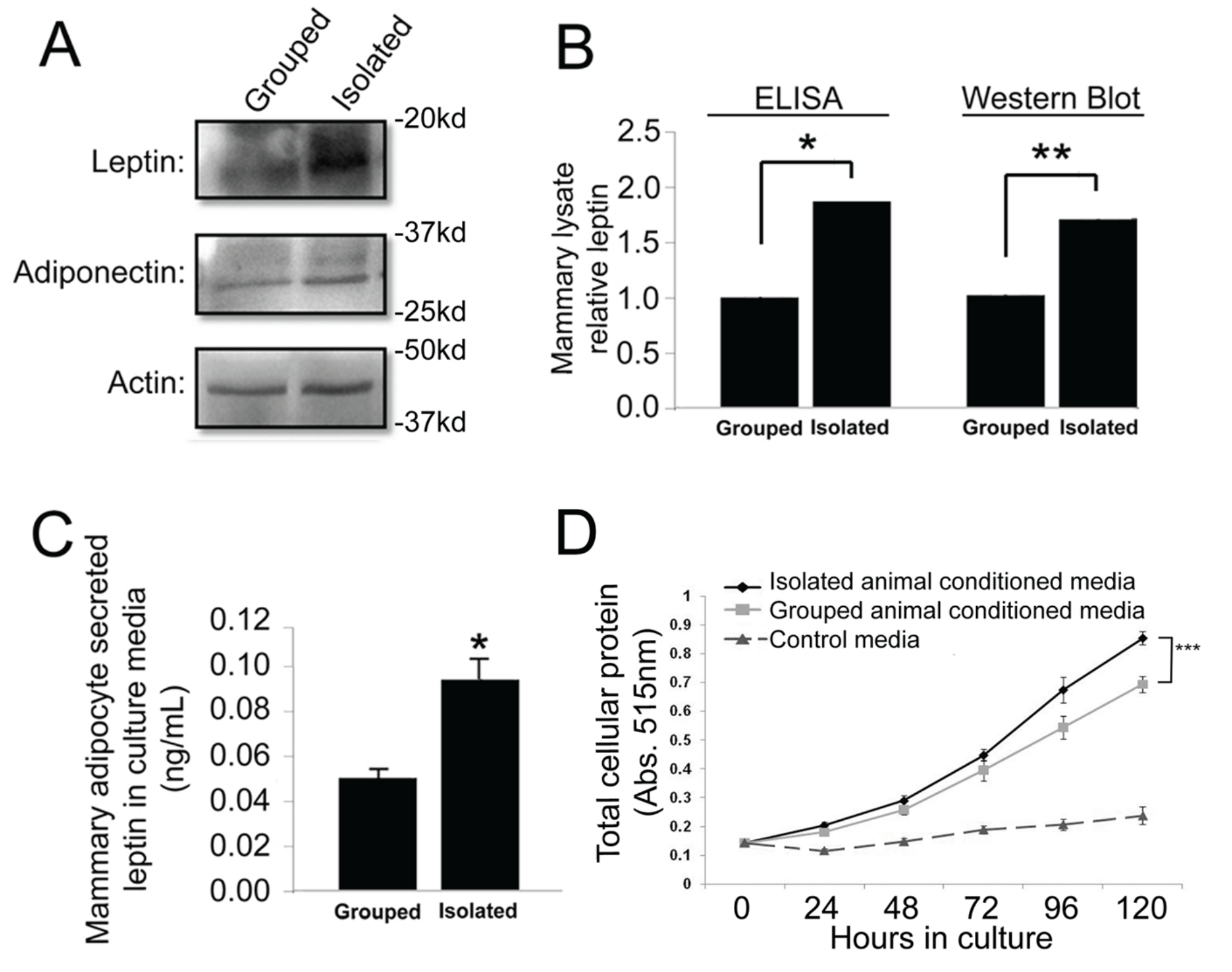

A
100 um
100 um
B
Relative (fold) gene expression
(adipocytes vs. non-adipocytes)
80
60
40
20
1
0
HK2
ACLY
ACACA
C
Relative gene expression in adipocytes
(isolated vs. group housed mice)
3.5
3
2.5
2
1.5
1
0.5
0
*
***
***
HK2
ACLY
ACACA
D
Relative gene expression in non-adipocytes
(isolated vs. group housed mice)
3.5
3
2.5
2
1.5
1
0.5
0
HK2
ACLY
ACACA
E
Relative gene expression in gonadal fat
(isolated vs. group housed mice)
3.5
3
2.5
2
1.5
1
0.5
0
HK2
ACLY
ACACA


A
Relative mammary gland gene expression (isolated vs. group CD1 mice)
0
1
2
3
4
5
6
7
8
9
**
*
HK2
ACLY
ACACA
B
Relative mammary gland gene expression (isolated vs. group WT FVB/N mice)
0
1
2
3
4
5
*
***
*
HK2
ACLY
ACACA
C
Relative gonadal fat gene expression (isolated vs. group CD1 mice)
0
1
2
3
4
5
6
7
8
9
HK2
ACLY
ACACA
D
Relative gonadal fat gene expression (isolated vs. group WT FVB/N mice)
0
1
2
3
4
5
*
HK2
ACLY
ACACA


A
glucose
Adapted from Williams et al., 2009 (5)
glucose
HK2
glucose-6-P
glycolysis
pyruvate
TCA cycle
ACLY
ACACA
citrate
acetyl-CoA
Malonyl-CoA
lipids
lipogenesis


B
Relative glucose consumption
7
6
5
4
3
2
1
0
Grouped
Isolated
*
*
Basal
+Insulin


C
Relative lipogenesis
14
12
10
8
6
4
2
0
Grouped
Isolated
*
*
Basal
+Insulin


A
Grouped
Isolated
Leptin:
-20kd
Adiponectin:
-37kd
-25kd
Actin:
-50kd
-37kd
B
ELISA
Western Blot
Mammary lysate relative leptin
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Grouped
Isolated
Grouped
Isolated
C
Mammary adipocyte secreted leptin in culture media (ng/mL)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
Grouped
Isolated
D
Isolated animal conditioned media
Grouped animal conditioned media
Control media
Total cellular protein (Abs. 515nm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
0
24
48
72
96
120
Hours in culture

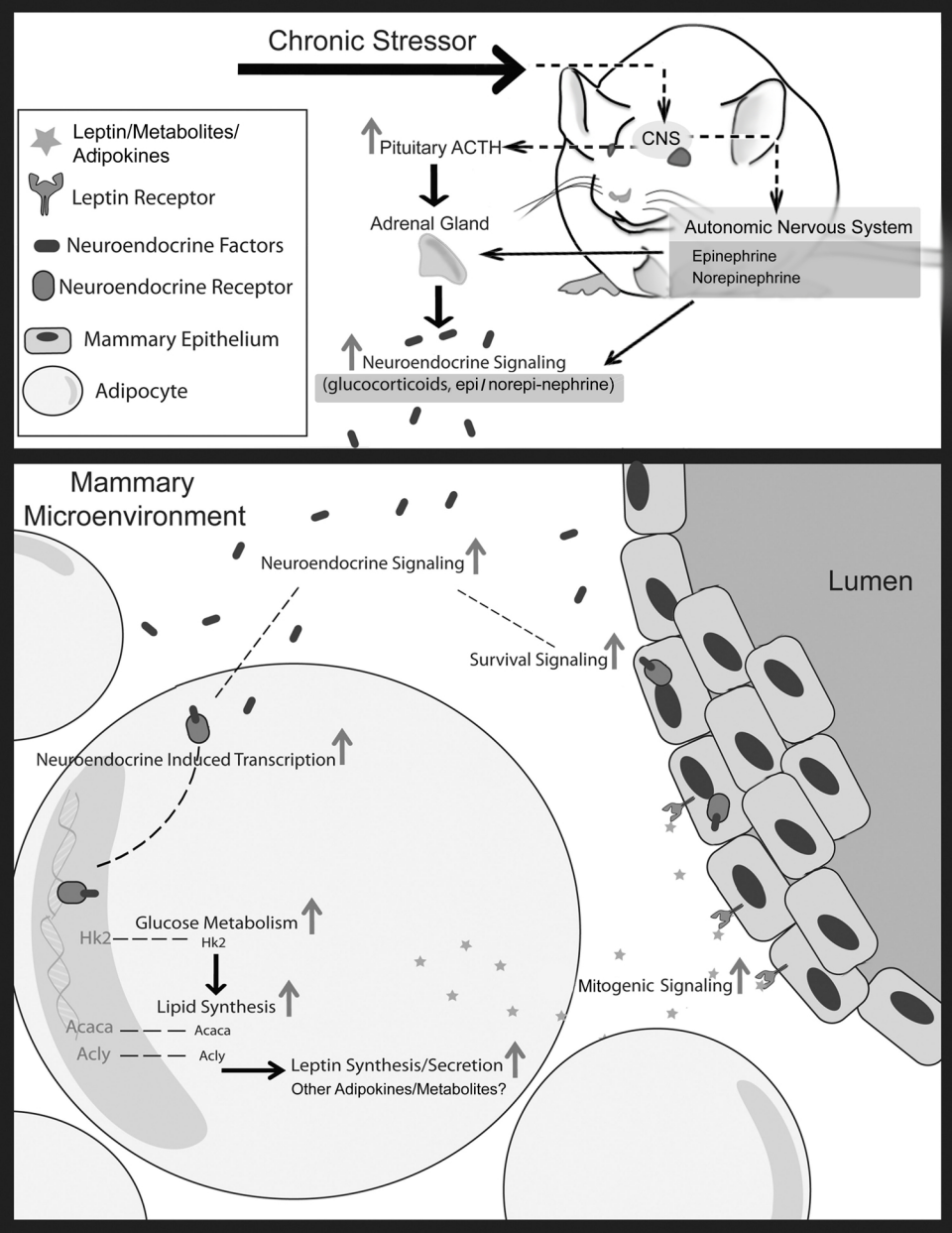

Chronic Stressor
Leptin/Metabolites/ Adipokines
Leptin Receptor
Neuroendocrine Factors
Neuroendocrine Receptor
Mammary Epithelium
Adipocyte
Pituitary ACTH
CNS
Adrenal Gland
Autonomic Nervous System
Epinephrine
Norepinephrine
Neuroendocrine Signaling
(glucocorticoids, epi / norepi-nephrine)
Mammary Microenvironment
Neuroendocrine Signaling
Lumen
Survival Signaling
Neuroendocrine Induced Transcription
Glucose Metabolism
Hk2
Hk2
Lipid Synthesis
Acaca
Acaca
Acly
Acly
Leptin Synthesis/Secretion
Other Adipokines/Metabolites?
Mitogenic Signaling

Table 1: Measurements of circulating metabolic parameters, food consumption, and weights in grouped vs. isolated TAg female mice.

	Grouped	Isolated	Pvalue
Blood glucose (mg/dL)	113.4 +/- 9.85	110.4 +/- 3.54	0.79
Serum Insulin (pg/mL)	361.5 +/- 78.8	349.3 +/- 82.8	0.92
NEFA (mEq/L)	0.93 +/- 0.10	0.79 +/- 0.07	0.24
Serum Leptin (ng/mL)	1.36 +/- 0.39	1.31 +/- 0.55	0.94
Serum Cort. (ng/mL)	76.8 +/- 37.0	67.9 +/- 39.3	0.66
Food consumption: 8-10 wks (kcal/day)	9.33 +/- 0.36	10.60 +/- 0.26	0.03
Food consumption: 11-17 wks (kcal/day)	10.47 +/- 0.35	12.58 +/- 0.35	0.002
Weight: age 10 wks (g)	18.9 +/- 0.48	18.8 +/- 0.23	0.85
Weight: age 17 wks (g)	21.2 +/- 0.47	21.4 +/- 0.54	0.81

Appendices, section 2:

Figure Legends:

Fig. 1: Activation of glycolysis and lipid synthesis pathways in socially isolated animals' mammary adipose tissue. A, relative expression of metabolic genes in socially isolated animals' mammary fat (black bars) compared to group-housed animals' mammary fat (grey). B, Pathway map of metabolic genes profiled in mammary fat from grouped and socially isolated animals. Green dashed lines indicate putative ChREBP-α direct target genes based on ChIP analysis. Red asterisks indicate genes identified as being significantly increased in socially isolated animals.). Error bars indicate standard deviation; * p≤0.05, ** p≤0.01.

Fig. 2: Lipidomics profiling of polar lipids. A, Distribution of polar lipid classes identified in mammary fat of grouped vs. socially isolated TAg mice. B, Absolute quantification of total polar lipids in mammary fat of grouped vs. isolated mice.

Fig. 3: Specific polar lipid classes are increased in the mammary fat of socially isolated animals. Relative polar lipid concentrations for lipid classes profiled. Error bars indicate standard deviation; *p≤0.05 with Bonferroni correction.

Fig. 4: Specific neutral lipids are increased within the mammary fat of socially isolated animals. Relative neutral lipid concentrations in grouped vs. socially isolated TAg mice. * p≤0.05, ** p≤0.01.

Fig. 5: LPC is toxic to M6 breast cancer cells. M6 cells were incubated with the indicated doses of LPC 16:0 in the presence of absence of 2.5% FBS and percentage of dead cells was measured after 8hrs using YOYO-1 iodide staining.

Fig. 6: LPC in the presence of mammary fat conditioned media promotes M6 cell survival and proliferation. M6 cells were incubated with the indicated doses of LPC in the presence or absence of mammary fat conditioned media. Proliferation was measured as change in confluence with respect to time using the Incucyte™ live cell imaging system. Dead cells (YOYO-1 iodide positive) were counted using the Incucyte™ live cell imaging software and normalized to confluence. A, M6 cells' change in confluence with LPC doses and serum free conditions. B, dead cell count normalized to confluence with LPC doses and serum free conditions. C, M6 cells' change in confluence with LPC doses and mammary fat conditioned media. B, dead cell count normalized to confluence with LPC doses and mammary fat conditioned media.

Fig. 7: Mammary fat conditioned media contains phospholipase-D activity. BNPP was added to serum free media and mammary fat conditioned media. Conversion of BNPP

to PNP, an indication of phospholipase-D mediated BNPP cleavage, was determined by yellow product formation and 410nm absorbance.

Fig. 8. LPA promotes survival and proliferation of TAg breast cancer cells under serum free conditions. A, proliferation of M6 breast cancer cells in serum free media with increasing concentrations of LPA 18:1. B, Count of dead cells (YOYO-1 iodide positive) cultured under serum-free conditions with increasing concentrations LPA 18:1

Table 1: Top 20 significantly changed polar lipids ranked according to P-value.

Table 2: Concentrations of neutral lipid classes in grouped vs. socially isolated TAg mice.

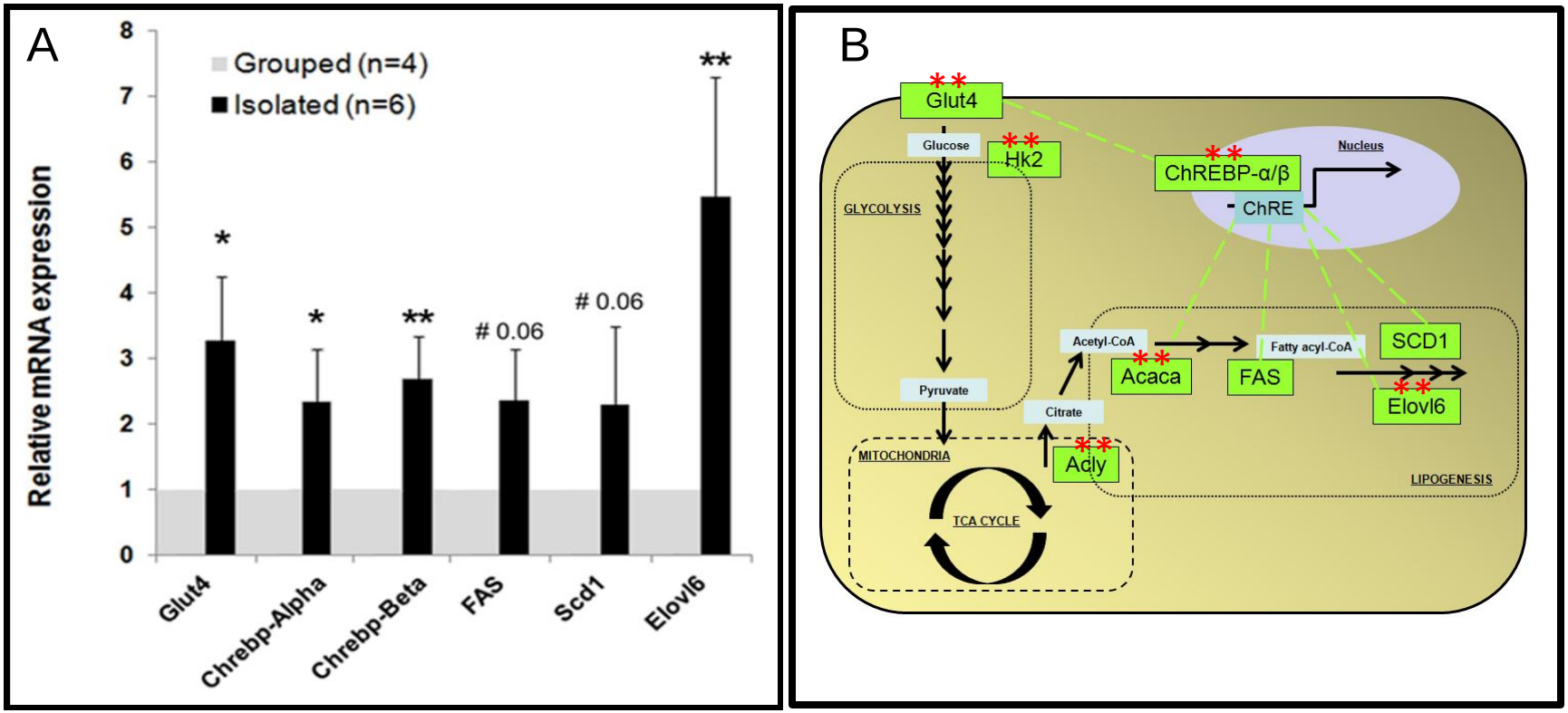

A
Relative mRNA expression
Grouped (n=4)
Isolated (n=6)
0
1
2
3
4
5
6
7
8
*
*
**
0.06
0.06
**
Glut4
Chrebp-Alpha
Chrebp-Beta
FAS
Scd1
Elovl6
B
Glut4
Glucose
Hk2
GLYCOLYSIS
Pyruvate
MITOCHONDRIA
TCA CYCLE
Citrate
Acly
Acetyl-CoA
Acaca
Fatty acyl-CoA
FAS
SCD1
Elovl6
LIPOGENESIS
Nucleus
ChREBP-α/β
ChRE

Figure 1.

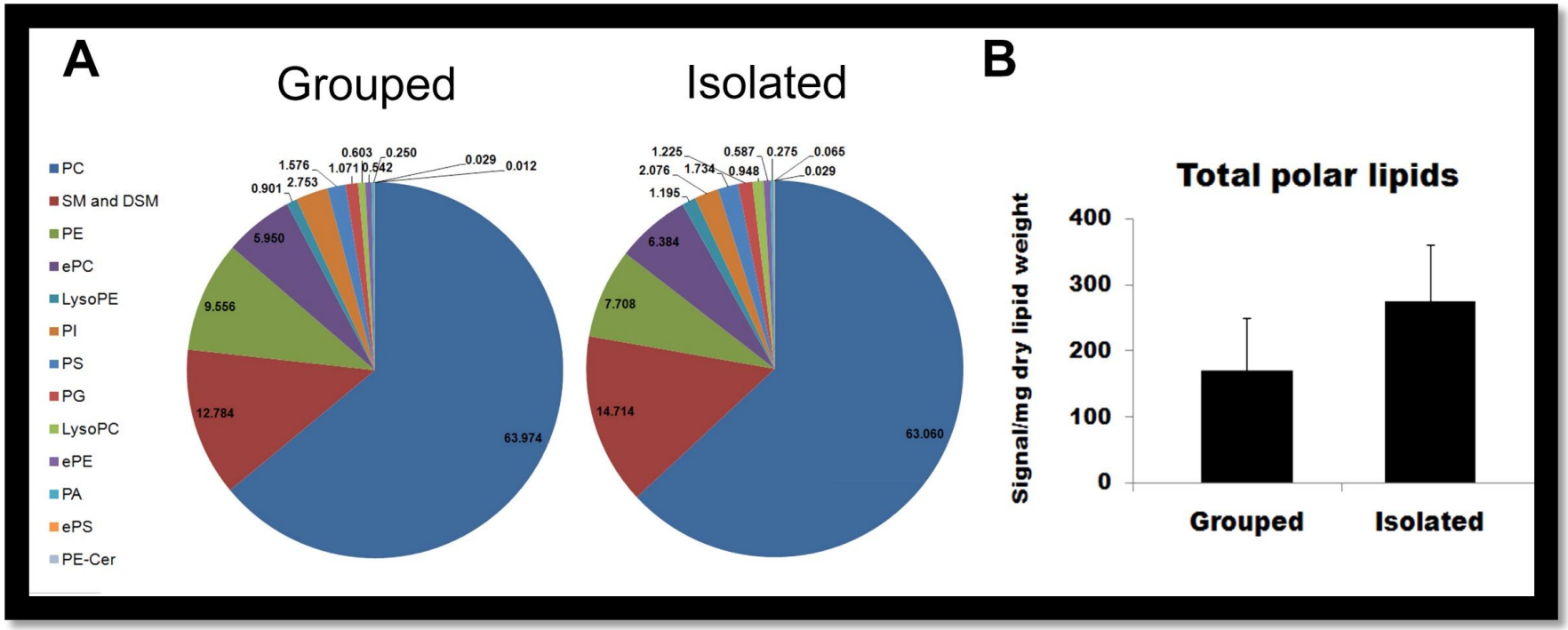

A
Grouped
Isolated
PC
SM and DSM
PE
ePC
LysoPE
PI
PS
PG
LysoPC
ePE
PA
ePS
PE-Cer
63.974
12.784
9.556
5.950
0.901
2.753
1.576
1.071
0.603
0.542
0.250
0.029
0.012
63.060
14.714
7.708
6.384
1.195
2.076
1.734
1.225
0.948
0.587
0.275
0.065
0.029
B
Total polar lipids
Signal/mg dry lipid weight
400
300
200
100
0
Grouped
Isolated

Figure 2.

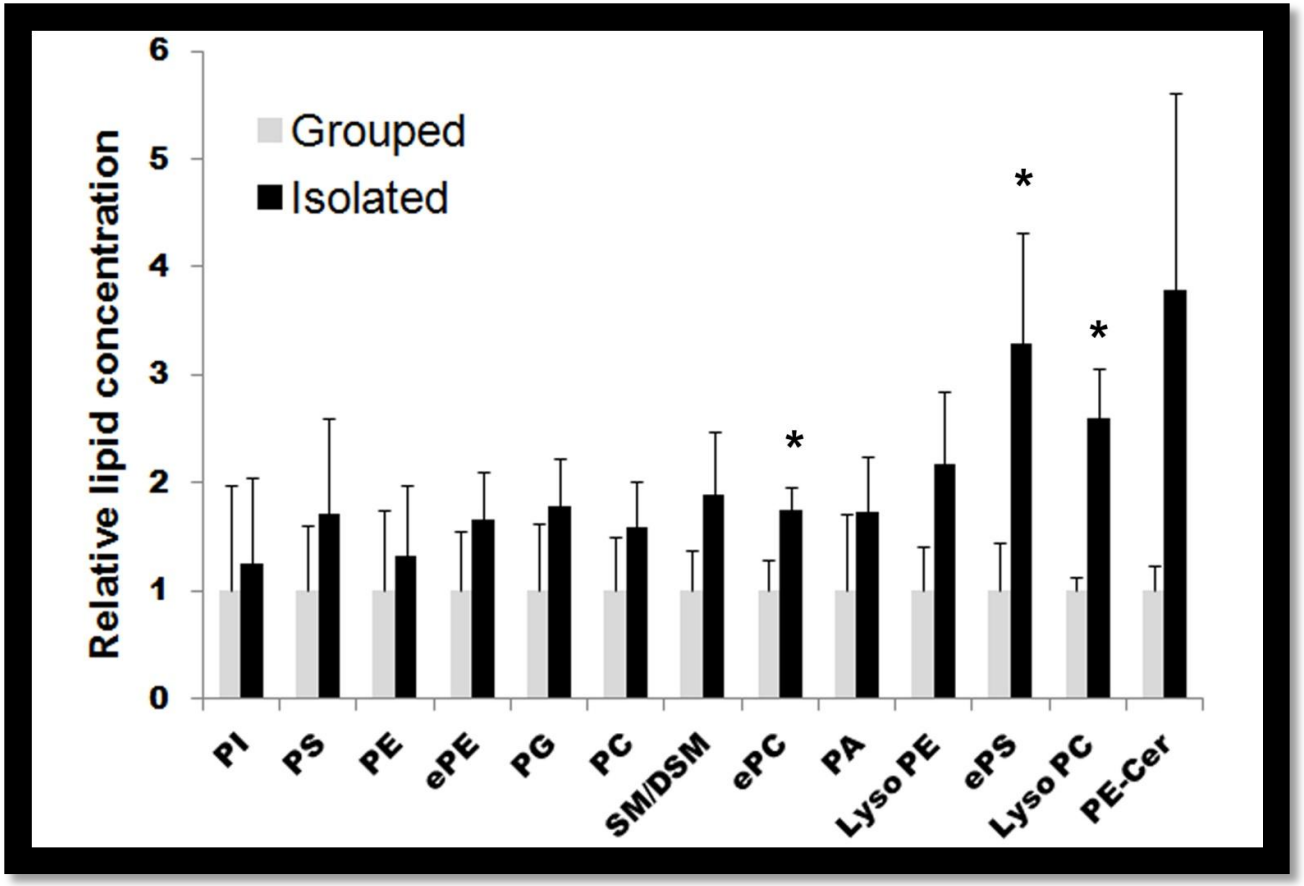

Relative lipid concentration
6
5
4
3
2
1
0
Grouped
Isolated
*
*
*
PI
PS
PE
ePE
PG
PC
SM/DSM
ePC
PA
Lyso PE
ePS
Lyso PC
PE-Cer

Figure 3.

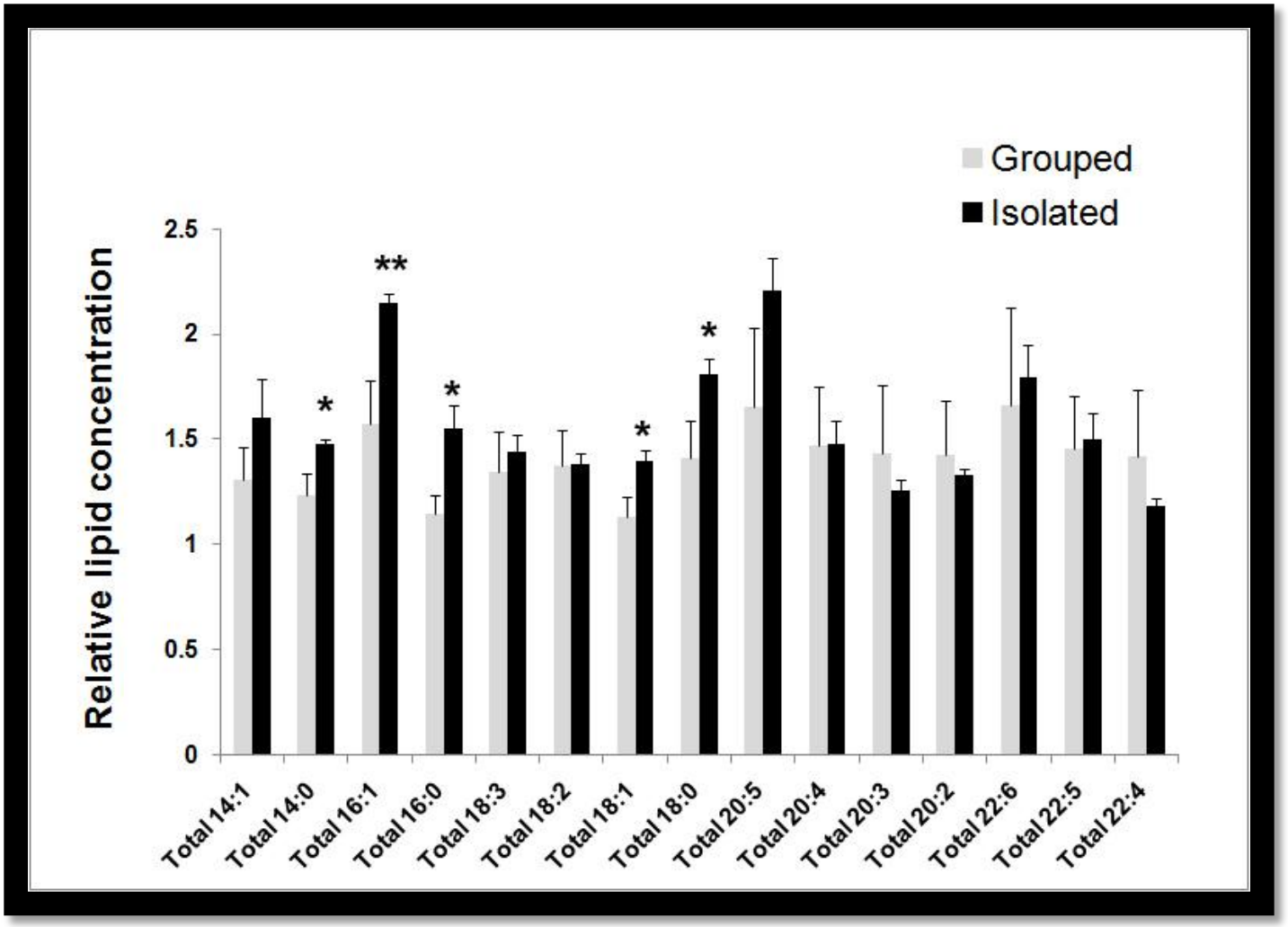

Grouped
Isolated
Relative lipid concentration
2.5
2
1.5
1
0.5
0
*
**
*
*
*
Total 14:1
Total 14:0
Total 16:1
Total 16:0
Total 18:3
Total 18:2
Total 18:1
Total 18:0
Total 20:5
Total 20:4
Total 20:3
Total 20:2
Total 22:6
Total 22:5
Total 22:4

Figure 4.

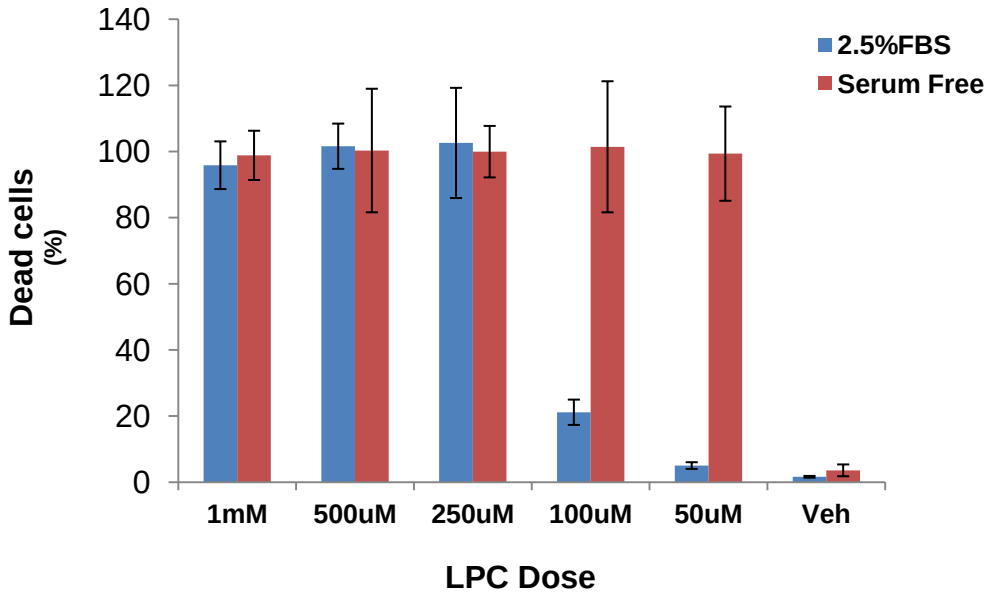

140
120
100
80
60
40
20
0
Dead cells (%)
2.5%FBS
Serum Free
1mM
500uM
250uM
100uM
50uM
Veh
LPC Dose

Figure 5.

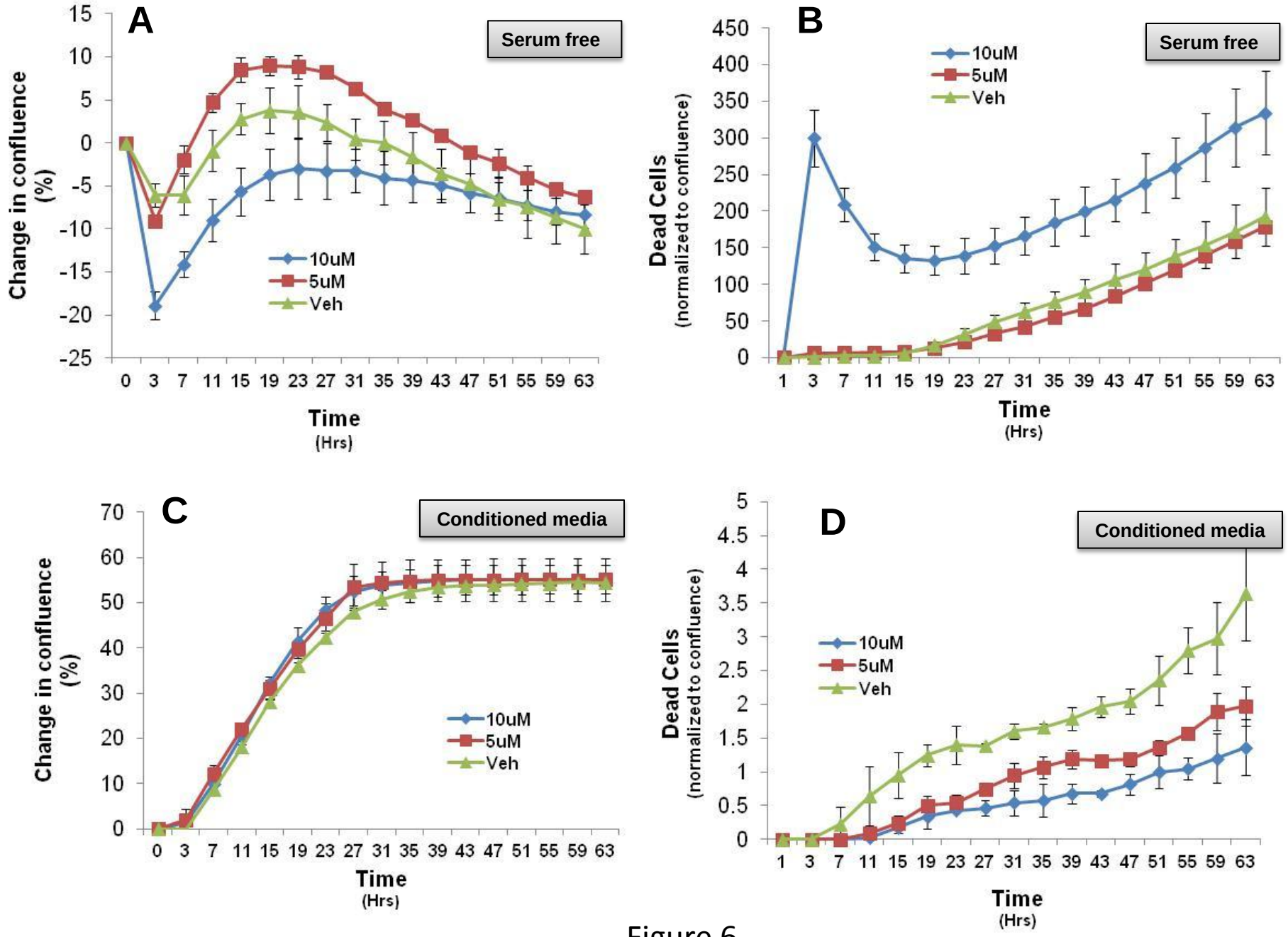

A
Serum free
Change in confluence (%)
15
10
5
0
-5
-10
-15
-20
-25
0 3 7 11 15 19 23 27 31 35 39 43 47 51 55 59 63
Time (Hrs)
10uM
5uM
Veh
B
Serum free
Dead Cells (normalized to confluence)
450
400
350
300
250
200
150
100
50
0
1 3 7 11 15 19 23 27 31 35 39 43 47 51 55 59 63
Time (Hrs)
10uM
5uM
Veh
C
Conditioned media
Change in confluence (%)
70
60
50
40
30
20
10
0
0 3 7 11 15 19 23 27 31 35 39 43 47 51 55 59 63
Time (Hrs)
10uM
5uM
Veh
D
Conditioned media
Dead Cells (normalized to confluence)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1 3 7 11 15 19 23 27 31 35 39 43 47 51 55 59 63
Time (Hrs)
10uM
5uM
Veh

Figure 6.

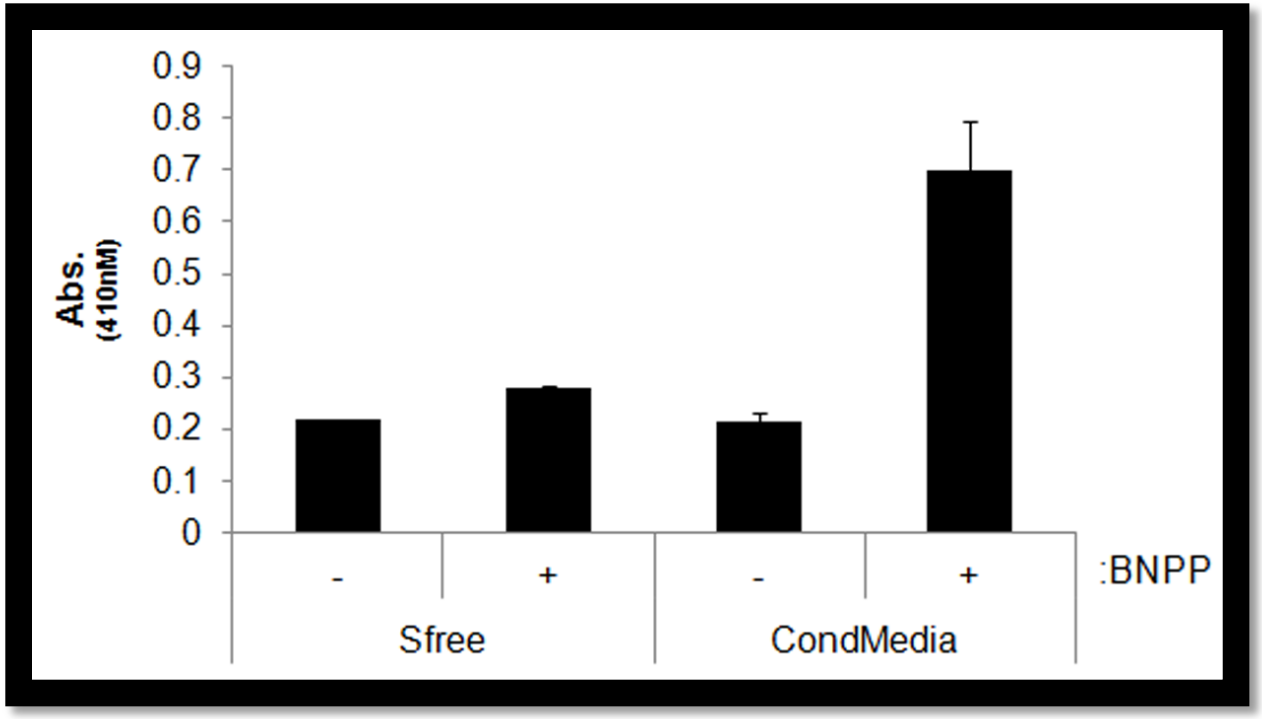
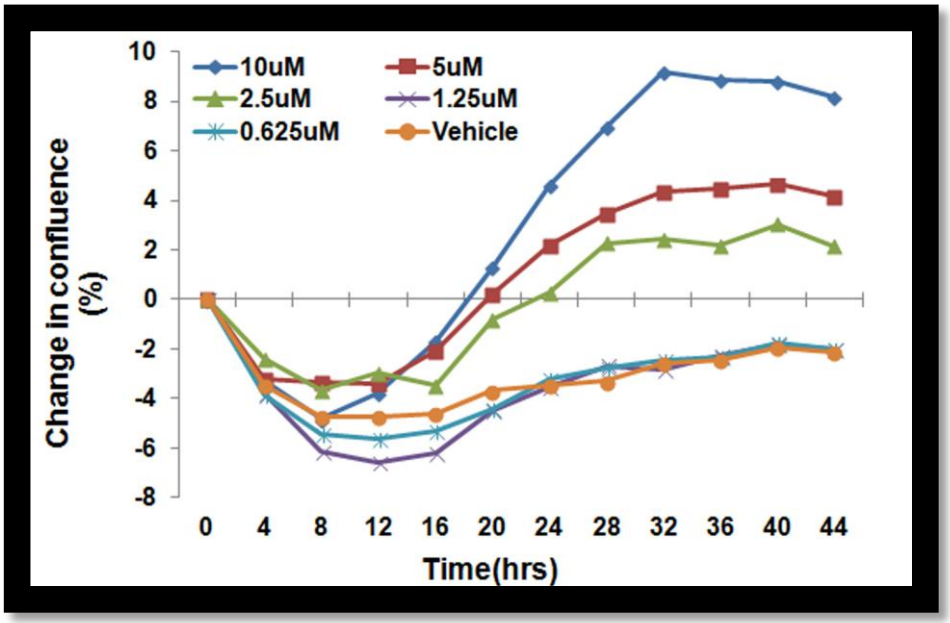


Figure 7.


10uM
5uM
2.5uM
1.25uM
0.625uM
Vehicle
Change in confluence (%)
10
8
6
4
2
0
-2
-4
-6
-8
0
4
8
12
16
20
24
28
32
36
40
44
Time(hrs)

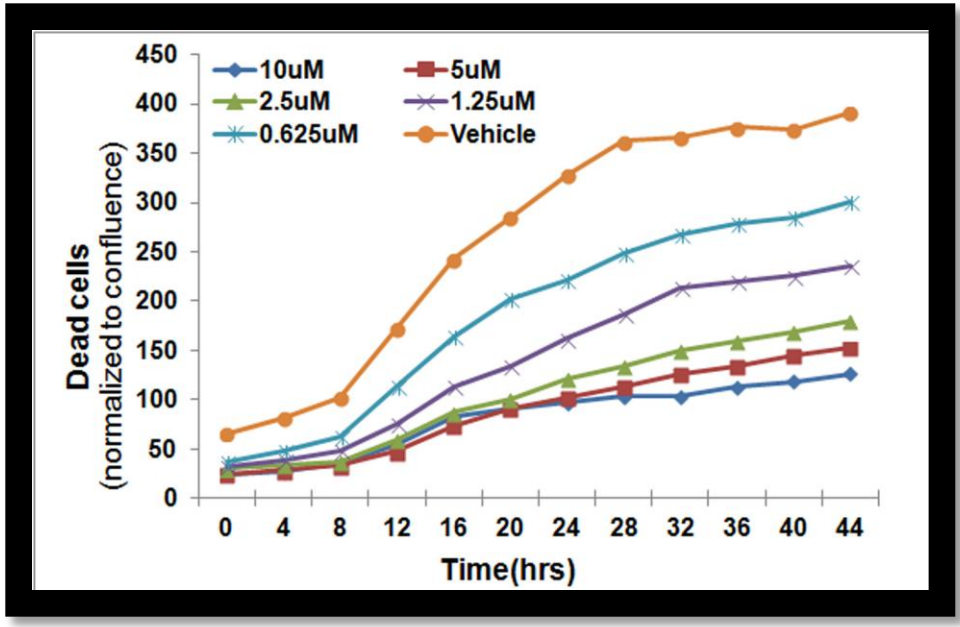

10uM
5uM
2.5uM
1.25uM
0.625uM
Vehicle
Dead cells (normalized to confluence)
450
400
350
300
250
200
150
100
50
0
0
4
8
12
16
20
24
28
32
36
40
44
Time(hrs)

Figure 8.

Rank	Mass	Compound Formula	Compound Name	p-val
1	496.3	C24H50O7PN	**LPC(16:0)**	0.0001490
2	870.7	C50H96O8PN	**PC(42:2)**	0.0001551
3	734.5	C38H72O10PN	**PS(32:1)**	0.0001824
4	522.3	C26H52O7PN	**LPC(18:1)**	0.0002745
5	480.3	C23H46O7PN	**LPE(18:1)**	0.0003017
6	494.3	C24H48O7PN	**LPC(16:1)**	0.0003116
7	840.6	C48H90O8PN	**PC(40:3)**	0.0005223
8	818.6	C44H84O10PN	**PS(38:1)**	0.0005362
9	828.6	C47H90O8PN	**PE(42:2)**	0.0006506
10	798.6	C45H84O8PN	**PE(40:3)**	0.0006913
11	524.4	C26H54O7PN	**LPC(18:0)**	0.0007365
12	814.5	C47H76O8PN	**PE(42:9)**	0.0009465
13	664.5	C35H70O8PN	**PE(30:0)**	0.0010830
14	802.6	C44H84O9PN	**ePS(38:2)**	0.0013139
15	712.5	C39H70O8PN	**PE(34:4)**	0.0013176
16	788.6	C44H86O8PN	**PC(36:1)**	0.0013196
17	862.6	C48H80O10PN	**PS(42:7)**	0.0014234
18	948.5	C51H79O13P	**PI(42:10)**	0.0016986
19	822.6	C48H88O7PN	**ePC(40:5)**	0.0017553
20	850.6	C49H88O8PN	**PE(44:5)**	0.0017829

Table 1.

	Grouped	Isolated	Pvalue
Total 14:1	1.359026	1.661565	0.219989
Total 14:0	28.37567	33.98356	0.022288
Total 16:1	69.50874	94.73596	0.007754
Total 16:0	358.9951	486.9224	0.012314
Total 18:3	20.59789	22.04222	0.587854
Total 18:2	287.045	288.5631	0.960902
Total 18:1	403.9124	499.0571	0.020276
Total 18:0	110.649	142.2067	0.035092
Total 20:5	1.294511	1.724969	0.133205
Total 20:4	3.041031	3.052704	0.982378
Total 20:3	1.667471	1.457382	0.49942
Total 20:2	2.269884	2.123427	0.66278
Total 22:6	2.229097	2.415044	0.73185
Total 22:5	1.032384	1.066587	0.840775
Total 22:4	0.123311	0.10279	0.369624

Table 2.